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INTRODUCTION

In addition to being the leading cause of death for civilians under 45 years of age, recent studies have confirmed that traumatic brain injury (TBI) is one of the most frequent causes of morbidity and mortality on the modern battlefield. Specifically, 40% of battlefield fatalities in the Viet Nam war were due to head wounds. It has been reported that of patients arriving alive at military field hospitals, 20% with extremely severe brain wounds die before surgery was performed, and 80% received neurosurgical treatment, with a 10% surgical mortality rate. Penetrating head injury alone accounts for 25% of all wartime casualties and approximately 40% of these injuries are fatal. Recently, studies of casualties during the Iraqi Conflict suggest that as many as 2/3 of combat casualties have associated concussive brain injuries.

Thus, the current proposal focuses on development of non-invasive diagnostics of TBI that ultimately will be useful in a battlefield environment. The research has been divided into 3 SOWs. The SOWs are as follows:

SOW 1: To employ integrated proteomics-based technologies to identify specific proteins or peptide fragments in brain released into CSF and/or blood of rats following experimental traumatic brain injury (TBI) or focal cerebral ischemia (middle cerebral artery occlusion: MCAO).

(A) Conduct concurrent studies employing mass spectrometry (HPLC MALDI-TOF protein profiling's Isotope-coded affinity tags-ICAT), 2D-gel electrophoresis, and phage display of single chain antibodies to detect proteins or peptide fragments in brain and CSF after TBI or MCAO.

(B) Employing injury-related proteins or peptide fragments identified in SOW 1-A, construct and validate the sensitivity of an antibody chip. Validation of the chip would on focus studies in CSF but would also explore chip utility for blood analyses.

SOW 2: Employing the antibody chip developed in SOW 1-B, determine which protein or peptide fragments released into CSF following TBI or MCAO are reliably associated with different injury magnitudes and predict changes in histopathological, behavioral and electrophysiological outcome measures.

(A) CSF will be sampled at multiple time points following injury to determine the optimal sampling time(s) predictive of injury magnitudes. If feasible based on data from SOW 1-B, limited studies will be conducted employing blood samples. (Months 25-30).

(B) Using the same injury magnitudes and data on release of protein or peptide fragments derived from the antibody chips employed in SOW 2-A, identify which sampling time(s) and which protein or peptide fragments released into CSF are optimally predictive of histopathological behavioral or electrophysiological assessments of outcome following TBI or MCAO. EEG analyses of electrophysiological alterations will be conducted following TBI and ischemia. Histopathology will be assessed by hematoxylin and eosin (H&E) staining for TBI and triphenyltetrazolium chloride (TTC) staining for MCAO. Behavioral assessments will include Morris water maze and Rotorod assessments following TBI and neurological examinations and forelimb sensorimotor assessments following MCAO.

SOW 3:

(A) Develop highly sensitive, quantitative ELISA-based assays capable of detecting blood levels of protein or peptide fragments determined to be optimally predictive of injury magnitude and outcome in SOW 2.
(B) Conduct preliminary validation of the utility of ELISA based assays employing blood samples taken following brain injury.

BODY

We have confirmed that accumulation of calpain and caspase-3 proteolytic fragments of brain derived αII-spectrin accumulate in CSF after middle cerebral artery occlusion (MCAO) in rats (Pike et al., JCBFM, 2004). This investigation confirmed previous studies in TBI that accumulation of calpain and caspase-3-cleaved-αII-SBDPs accumulate in CSF of rodents following MCAO. Following MCAO injury, full length αII-spectrin protein was decreased in brain tissue and increased in CSF. Calpain and caspase-3 αII SBDPs were also increased in brain and CSF after injury. Levels of these proteins were undetectable in CSF of uninjured control rats. These results indicate that calpain and caspase-3 cleaved αII SBDPs in CSF may be useful diagnostic indicators of cerebral infarction that could provide important information about specific neurochemical events that occur in the brain after acute stroke. In addition, these data confirm that similar profiles occur following stroke as well as TBI.

We have recently completed studies and are preparing a manuscript that confirms that αII-SBDPs accumulating in CSF after TBI are associated with injury magnitude and predict lesion size. This study used immunoblotting to measure levels of SBDP in contused brain and CSF within 24 hrs after mild and more severe cortical impact brain injury. CSF SBDP levels were correlated with lesion volumes calculated using t2 weighted MRI, and lesion sizes were then correlated with neuromotor deficits. The utility of SBDP as a biomarker was compared to other putative biomarkers such as cleaved tau and S-100B. αII-SBDPs were clearly superior to S-100B as biomarkers. These important studies confirm the potential clinical utility of biomarkers such as αII-SBDP and lay the foundation for future validation of novel biomarkers discovered by this research platform (manuscript in preparation).

We have 2 important reviews that are of relevance to investigators developing biomarkers for acute CNS injury. The first review summarizes the status of research on biomarkers of proteolytic damage following TBI (Pineda, et al, in press). The second review (Denslow, et al, J Neurotrauma, 2003) provides an important resource for application of proteomics technology to the study of acute CNS injury. These 2 reviews confirm the leading role our research laboratory is taking in this new and important field.

KEY RESEARCH ACCOMPLISHMENTS

- Following up on original work done in TBI, we have confirmed that breakdown products of αII-spectrin are also released into the cerebrospinal fluid (CSF) of rats following MCAO.
- We have initiated studies to confirm that biochemical markers of TBI are associated with varying magnitudes of injury, including lesion volume.
- We have reviewed the current status of biochemical markers of acute brain injury in a peer reviewed paper.
- We have summarized the application of proteomics technologies for studies of acute brain injury in a peer reviewed publication.

- We have developed an effective integrated proteomics-based research platform that was an essential and necessary goal of the first year of funding.

REPORTABLE OUTCOMES

N. Denslow, M.E. Michel, M.D. Temple, C.Y. Hsu, K. Saatman and **R.L. Hayes**. Application of proteomics technology to the field of neurotrauma: Report from the human proteome project meeting. J. Neurotrauma 20(5): 401-407, 2003.

Brian R. Pike, Jeremy Flint, Jitendra R. Dave, X.-C. May. Lu, Kevin K.W. Wang, Frank C. Tortella and **Ronald L. Hayes**. Accumulation of calpain and caspase-3 proteolytic fragments of brain-derived αII-spectrin in CSF after middle cerebral artery occlusion in rats. JCBFM, 24(1): 98-106, 2004.

Jose A. Pineda, Kevin K.W. Wang, Ronald L. Hayes. Biomarkers of proteolytic damage following traumatic brain injury. Brain Pathology (In press).

NC Ringger, BE O'Steen, JG Brabham, X Silver, J Pineda, KKW Wang, RL Hayes. A novel biomarker for traumatic brain injury: CSF alpha-II-spectrin breakdown product levels are associated with injury magnitude and predict lesion size. (In preparation)

CONCLUSIONS

In summary, we have made strong progress within the first 12 months of our funding. We have developed an effective, integrated proteomics based platform to study biomarkers in TBI and ischemia (MCAO). We have confirmed and published that MCAO, like TBI, is a useful model of biomarker discovery. We have established a powerful, proteomics-based platform that will yield important new data over the next year.

UNIVERSITY OF FLORIDA

APPENDIX

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ANNUAL REPORT

ON

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Biochemical Markers of Brain Injury: An Integrated Proteomics Based Approach

PI: Ronald L. Hayes, PhD

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Application of Proteomics Technology to the Field of Neurotrauma

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ABSTRACT

Near-completion of the Human Genome Project has stimulated scientists to begin looking for the next step in unraveling normal and abnormal functions within biological systems. Consequently, there is new focus on the role of proteins in these processes. Proteomics is a burgeoning field that may provide a valuable approach to evaluate the post-traumatic central nervous system (CNS). Although we cannot provide a comprehensive assessment of all methods for protein analysis, this report summarizes some of the newer proteomic technologies that have propelled this field into the limelight and that are available to most researchers in neurotrauma. Three technical approaches (two-dimensional gel electrophoresis, direct analysis by mass spectrometry, including two-dimensional chromatography coupled to mass spectrometry and isotope coded affinity tags, and antibody technologies) are reviewed, and their advantages and disadvantages presented. A discussion of proteomic technology in the context of brain and spinal cord trauma follows, addressing current and future challenges. Proteomics will likely be very useful for developing diagnostic predictors after CNS injury and for mapping changes in proteins after injury in order to identify new therapeutic targets. Neurotrauma results in complex alterations to the biological systems within the nervous system, and these changes evolve over time. Exploration of the "new nervous system" that follows injury will require methods that can both fully assess and simplify this complexity.

Key words: brain trauma; mass spectroscopy; proteomics; spinal cord trauma

INTRODUCTION

THE MAPPING OF THE HUMAN GENOME has provided new technologies and theories necessary to tackle evaluation of very complex biological systems. Assessment of genetic information and the interactions of genes with environmental influences will advance understanding of basic neurobiology, pathophysiology of disease states, and potential therapies. However, genetics cannot completely answer the questions that arise in studying injury in the nervous system. Indeed in a variety of fields, scientists criticize the use of genomics as a tool, because DNA sequencing provides only a snapshot of the different ways a cell may use its genes. Any cell constantly reacts to its

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changing environment, creating a dynamic system (Persidis, 2000), and there seems to be a low correspondence ($R^2 = 0.61$) between changes at the transcription level and changes at the proteomic level (Ideker et al., 2001). Although proteomics is in some ways a "catch-all" term, it conceptualizes the many complex interactions that occur in the cellular repertoire for proteins. These events include genome-coded processes and posttranslational modifications, as well as interactions among proteins, nucleic acids, lipids, and carbohydrates (Persidis, 2000).

As of 2001, the Human Genome Project estimated that there are only about 30,000 genes (Claviere, 2001; Venter et al., 2001). Although the final figure is currently under debate (Shouse, 2002), this relatively low number of genes suggests greater roles for regulation of RNA translation and posttranslational modifications of resulting proteins in the cellular response to normal and pathological stimuli. Researchers have been investigating individual proteins or protein families in basic biology and disease for decades, but the field of proteomics offers a more global perspective. Because such analysis embraces the study of large numbers of inter-related proteins and their involvement in selected physiological or pathological states, a more robust appreciation of the injured nervous system may emerge.

This report serves as a beginning for the broad appreciation of the burgeoning field of proteomics. Here, we discuss the advantages and disadvantages of selected methodologies, and how they can be applied to study cellular events that occur after trauma to the brain or spinal cord. We will concentrate on the "gold standard" two-dimensional gel electrophoresis (2D GE) and a representation of emerging technologies for the field of neurotrauma that can be used in laboratories that do not have proteomics per se as a focus. The usefulness of proteomic investigation in neuroscience in general is currently under intense discussion (Grant, 2001; Grant and Blackstock, 2001), and the field of neurotrauma can position itself to embrace or reject findings as they emerge.

CURRENT AND FUTURE TECHNIQUES FOR PROTEOMIC ANALYSIS

Acknowledged challenges for proteomics research surround the analysis of complex mixtures of proteins to identify expression profiles after a physical or chemical stress, and these issues certainly apply in neurotrauma. It has been estimated that there are around 300,000 proteins in the human proteome and likely similar numbers in animal models. The large number of proteins relative to genes can result from alternative splicing of transcripts, direct protein modifications due to specific cleavages, or other post-translational events. Because so many potential proteins and multiple time points will be involved in any analysis of neural injury, approaches must be based on established biochemical principles and amenable to high throughput technologies. Techniques should allow sorting out of protein complexity, as well as analyzing and identifying proteins in low abundance or with atypical characteristics, such as basic or glycosylated proteins. Methods still need to be refined for studying protein–protein interactions, protein structure, and metabolic pathways. It is unlikely that one method of analysis can yield full information; most likely, a combination of different methods, each with its own specific advantages, will need to be employed. In addition, a major challenge will come in data storage and analysis, and biocomputing must be given extensive consideration whenever proteome-directed research is undertaken.

Two-Dimensional Gel Electrophoresis followed by Mass Spectrometric Identification of Proteins

This approach is the classic method for analyzing multiple proteins. Proteins are separated in the first dimension by isoelectric focusing (a property that depends on the relative amounts of acidic and basic amino acids in each protein) and in the second dimension by size. It is possible to see over 1,000 well-resolved proteins on a single gel. While the application of 2D GE is not new to neuroscience research (Amess and Tolkovsky, 1995; Buonocore et al., 1999; Charriaut-Marlangue et al., 1996), the new interest in this method is the ability to directly identify proteins that are differentially expressed by mass spectrometry (MS).

To identify proteins, each spot of interest is cut from the gel and digested with trypsin. The masses of the tryptic peptides are then obtained by MS (either MALDI TOF [Matrix-Assisted Laser Desorption Ionization Time-of-Flight] or LC-MS [Liquid Chromatography coupled to a Mass Spectrometer] and MS/MS) and are used to search databases for proteins that best match the experimental fragments obtained. With LC-MS/MS, it is possible to further subdivide the fragments and obtain amino acid sequence information which, when added to the database search, increases the chance of matching the protein.

Several improvements have made this method more robust and reproducible, including the discovery of better detergents and buffer combinations, new pH gradient strips, pre-cast SDS slab gels, more sensitive gel stains, and the development of difference gel electrophoresis (DIGE) (Unlu et al., 1997), which is now marketed freely as a kit by Amersham). The DIGE system involves a modification of the normal 2D gel method such that one is able to resolve both control and experimental samples in one gel. The two protein samples that are to be com-

pared, for example a control tissue and an injury tissue, are each pre-labeled with one of two cyanine dyes, Cy3 or Cy5. The labeled samples are mixed and co-migrated on the same gel in both dimensions, removing imperfections in the separation due to differences in the gel matrix, pH field, and other procedural effects. Because the samples are co-migrated, it is easy to quantify changes in protein expression and pick proteins that are altered by the treatment. The co-migration also reduces the number of gels that need to be performed for statistical purposes. The coupling of this method with mass spectrometry to identify proteins that are differentially expressed has recently been validated by Tonga et al. (2001) and is reviewed by Patton (2002).

These advances result in better resolution and visualization. Despite the improvements, 2D GE/MS remains technically complicated, and requires at least triplicate samples to be processed for statistical purposes. In addition, some classes of proteins are not detectable, including those that are rare (i.e., low abundance), small (i.e., under 2 kDa), glycosylated, of basic pH, or are integral membrane proteins. Table 1 lists the advantages and disadvantages to this method.

Overall Assessment of 2D GE

In general, 2D GE, especially DIGE, works well for protein discovery and is currently the most widely used technique for detecting proteins that have changed upon treatment. It is the only high-resolution method currently available to detect changes in post-translational modifications, including phosphorylation, which is critical to many proteins of significance to neuroscience. But 2D GE is still not able to detect the entire proteome, missing proteins that are present in low abundance and that have isoelectric points outside the normal range of pH 4–9. A substantial advancement would be the development of high-throughput affinity purification methods for low abundance proteins. Kits to affinity purify specific groups of proteins, for example phosphoproteins, are now available through some commercial vendors, and these have catapulted the study of differential protein expression. New techniques and improvements continually arise in this area; however, the need for specialized training of personnel is critical for 2D GE and MS.

Non–Gel-Based Separations of Proteins Coupled to Mass Spectrometry

This technical category is very broad and constantly evolving (Washburn et al., 2001). Methodologies include, but are not limited to, separations of protein digests by various chromatography procedures. Table 2 summarizes advantages and disadvantages of these methods. Several two-dimensional chromatography methods have been developed that separate proteins by orthogonal chromatography steps, for example, ion exchange followed by reverse phase high-performance liquid chromatography (HPLC) (Washburn et al., 2001). Complex fractions can be further resolved by mass spectrometry (MS and MS/MS). For instance, advances with this technology enabled the identification of as many as 1,484 yeast proteins in a single experiment. This technology is particularly useful for comprehensive proteome projects, and specifically can be applied to identify proteins, for example, integral membrane proteins, that are normally missed by 2D GE.

Another innovative technology in this general area has been the development of isotope coded affinity tags (ICAT) (Gygi et al., 1999; Patton, 2002). A new, im-

Table 1. Two-Dimensional Gel Electrophoresis with Mass Spectrometry

Advantages	*Disadvantages*
Simultaneous high resolution of multiple proteins	Technical expertise required
Rapid comparison of multiple gels	Sample-to-sample variability
Ability to fluorescently label proteins and co-migrate them in the same gel	Multiple controls/experiimental samples necessary
Improved reagents available	Many rare proteins not visible
Compatible with pre-fractionation of samples (enriches rarer proteins)	Some classes of proteins not detectable (e.g., <2 kDa, basic, glycosylated)
Mass spectrometry identifies lower amounts of protein	Integral membrane proteins are underrepresented in gels
Identify some post-translational modifications	
High-throughput capability	
Discovery of new, differentially expressed proteins	

TABLE 2. NON–GEL-BASED MASS SPECTROMETRY METHODS

Advantages	*Disadvantages*
No two-dimensional gel electrophoresis for initial separation	Requires high-resolution separation methods
Direct comparison of complex protein samples	For ICAT method, proteins without cysteine residues missed (10–15%)
High-throughput capability	Unlikely to identify post-translational modifications
Analyze wider range of protein concentrations	Requires sophisticated mass spectrometers and skill in running them
Discover novel/differentially expressed proteins and peptides	Commercially available reagents are very expensive
Analyses of proteins that are missed by two-dimensional gel electrophoresis (basic, acidic, small, integral membrane proteins)	

proved version of these tags, named cleavable ICAT, is now available in kit form from Applied Biosystems. Briefly, ICAT refers to a pair of affinity directed reagents, which differ from each other by nine mass units (a difference of nine ^{13}C-groups in the linker portion of the tag), and that target cysteine residues in proteins. A pair of reagents is used to tag the full proteome from control and treated cells (or tissue) with one or the other ICAT reagent, respectively. Every protein containing a cysteine residue would then be tagged appropriately. The reaction is highly specific and occurs with high efficiency. After tagging, the two protein groups are mixed and digested with trypsin. Fragments tagged by the reagents are separated on an avidin column, and the mixture is then further separated and analyzed by LC-MS/MS. Fragments generated from proteins in equal abundance in the two tissues will be only nine mass units apart (or multiples of nine, if the fragment contains more than one Cys), and of equal height in the mass spectrometer. Fragments from proteins that are differentially expressed will appear to be higher (or lower) than their corresponding partners. These fragments can then be targeted for MS sequencing. Identities of the parent compounds can be obtained by comparing the sequences to databases. While this method is outstanding for determining the amount of protein in relation to a treatment or injury, it is not useful for the detection of changes due to post-translational modifications. Because the protein is identified solely on the basis of MS/MS fragmentation of a single peptide, ICAT analysis works best with mass spectrometers that have high resolution and mass accuracy.

Overall Assessment of Direct Mass Spectrometry Methods

Non–gel-based mass spectrometry methods for identifying differentially expressed proteins have great potential. These methods depend on high-resolution chromatography to separate complex mixtures of proteins prior to mass spectrometry, requiring capillary chromatography for sensitivity and high-resolution mass spectrometry for identification of proteins. In addition, new advances in bioinformatics are required for complex analyses of very large data files that are generated by these procedures. New developments in all these areas appear frequently in the literature, and as these methods become more robust and routine they may enable direct proteome analyses.

ICAT has the potential to provide excellent data in a short time frame and is worth considering, if the proper mass spectrometer is available. Even though post-translational modifications may not be amenable to this analysis, it can yield information for proteins that are newly synthesized in response to a stressor. This method may be the technique of choice for analyzing "pull downs" or "immunoprecipitates" without having to analyze the samples first by gel electrophoresis. Importantly, ICAT and other direct methods of global protein analysis complement the 2D GE/MS method.

Protein and Antibody Chips

The concept of evaluating thousands of proteins at once by a chip-based method is very appealing; however, this technology is still in early stages of development and will require considerable work to arrive at a point where it can be widely used to assess global differential protein expression. There are, however, several commercial companies that are currently offering protein arrays (Table 3) and others that have them under development (ProteoMonitor, 2002). Many approaches are used, but two main types of chips are being investigated: antibody-based and protein-based. In the case of antibody-based chips, antibodies to proteins of interest are fixed on an

TABLE 3. COMPANIES CURRENTLY OFFERING PROTEIN MICROARRAYS

Company name and website	*Chip description*
Adaptive screening www.adaptive-screening.com	Recombinant protein array
BD Biosciences Clontech www.clontech.com	Antibody array; fluorescent technology
Discerna www.discerna.co.uk	*In situ* array; cell-free synthesis
Hypromatrix www.hypromatrix.com	Four array products and custom antibody array services
Jenni Array Technologies www.jenni.com	Peptide, small molecule, kinase substrate arrays
Luminex www.luminexcorp.com	Microsphere/bead; cell signaling and kinase activity
Molecular Staging www.molecularstaging.com	Antibody arrays; rolling circle amplification technology
Pepscan www.pepscan.nl	Peptide arrays anchored to glass slides
Zeptosens www.zeptosens.com	Six pre-spotted microarrays with recognition elements

Adapted from ProteoMonitor (www.proteomonitor.com). Total listing was 30 companies, many of which are still developing their products.

inert surface (i.e., glass slide, membrane, beads) and probed with the full complement of proteins from the tissue of interest. Antibody-based chips will be used primarily to measure differential expression. Protein-based chips rely on placing proteins directly on the surface, and would be used primarily to test for protein–protein or protein–drug interactions. There are advantages and disadvantages to chip technology. For antibody-based chips, advantages include the use of single-chain, Fv antibody phage libraries to rapidly find appropriate antibodies for a large number of antigens. For protein-based chips, a critical need will be the use of recombinant vectors (e.g., full length expression; LaBaer personal communication) for producing a full complement of proteins, including rare ones, for placement on a chip. Additional technical advantages are outlined in Table 4. However, disadvantages (Table 4) may likely include the need for prior knowledge of the protein (i.e., not ideal for protein discovery) as well as availability of high-affinity antibodies for the proteins to be examined.

Overall Assessment of the Chip and Antibody Method

This technology holds considerable promise for identifying differentially expressed proteins. Thus, it will be

TABLE 4. CHIP TECHNOLOGY

Advantages	*Disadvantages*
Measurement of hundreds of proteins in parallel	Prior knowledge of protein required
Antibodies of differing affinities can be on one chip	Necessary to have the antibodies
Possible use of very small samples (microdissection)	Antibody specificity is critical
Potential to distinguish post-translational modifications	Limited use with post-translational modifications (antibody must distinguish modified site)
Methods for amplifying signals under development	Dependent on antibody affinity and protein concentration

important to begin to consider how to build chips that may be of specific interest to CNS trauma. One starting point is the application of antibodies to proteins that are already known to be involved in CNS injury and/or repair. In the last two decades, an extensive body of literature has developed with respect to biochemical and cellular changes following traumatic injury to the brain and spinal cord. This knowledge could be a valuable starting point for developing "injury chips." In addition, studies using DNA microarrays could provide valuable information about changes at the mRNA level that may suggest additional proteins to evaluate. Although the technology is not standardized, starting to work in the area now could ensure that chips are developed that will be useful to the neurotrauma field in the future.

Bioinformatics

In order to achieve the best value of any method, full and meaningful analysis will be critical, and any proteomics approach will generate extensive data. It will be important to develop methods, or to apply methods developed by others, to interpret and categorize information in a useful manner. Approaches to analyzing proteomic data are already being addressed in a variety of fields. The use of large-scale proteomics technologies yielding proteome-wide maps to study expression or interaction will depend heavily on information storage, representation, and analysis. It is possible to access proteomics databases and software through the World Wide Web; however, the evolution of resources is very rapid. Trends and probable changes are discussed in a paper by Wojcik and Schachter (2000). Other investigators have also recognized the need for fast, accurate computational analysis of protein function, and have begun development of large-scale computational systems for the analysis of sequence and structure of proteins (Weir et al., 2001). In addition, researchers have employed searching algorithms to study proteomics spectra generated by mass spectroscopy (Petricoin et al., 2002). Investigators in CNS injury can usefully appropriate existing bioinformatics strategies and sculpt them to meet their specific needs.

DISCUSSION

The gold standard for protein determination is still two-dimensional gel electrophoresis (2D GE) followed by mass spectrometry (MS). This two-stage method allows for both hypothesis-driven and discovery-driven research strategies. New methods on the horizon include separations of proteins by non–gel-based methods followed by MS, methods that rely exclusively on molecular biology, and protein/antibody chips. Although both antibody-based and protein-based chips are now available commercially, development is at a very early stage and interpretations of any studies will be cautious. All of these methods permit parallel processing of several proteins at once and have the potential for increasing the sensitivity of detection to allow the use of small amounts of material. With improvements in such methods, rare proteins and cell type–specific proteins may be evaluated.

Although the study of proteins is not new, the field of "proteomics" is. In the past, several studies of CNS injury have used the high-resolution power of 2D gel electrophoresis (Amess and Tolkovsky, 1995; Buonocore et al., 1999; Charriaut-Marlangue et al., 1996; Jenkins et al. 2002; Leski and Steward, 1996). The work of Kirschenbaum and Pulsinelli (1990) performed over a decade ago to analyze differences in phosphorylation patterns in ischemic rat hippocampus, striatum, and neocortex tissues was extremely interesting and forward thinking for its time. Now with the coupling of MS to aid in the identification of proteins that are regulated, this 2D GE approach becomes even more powerful. Many of the newer mass spectrometric techniques can identify proteins down in the attomole (10^{-18} moles) to low femtomole (10^{-15} moles) levels.

The study of CNS trauma is a dynamic and exciting area of neuroscience, and because there is a readily defined event, dissection of complex neurobiological consequences can be attempted in this context. Information gained will provide insight into processes of cell death, regeneration, and plasticity that will have relevance to many other developmental or degenerative neurological disorders. There are limitations to current proteomic technologies, including minimal rapid high-throughput screening and detection of post-translational modifications, as well as issues regarding appropriate and meaningful bioinformatics/data analysis and interpretation. In neurotrauma, protein modifications will be important: injury initiates a complicated sequence of cellular events that affects many aspects of cell signaling, genome-coded events, protein–protein interactions, and post-translational processes. It is clear that proteomic technologies could have a large impact on the study of CNS injury. Information obtained may be extremely valuable for several reasons: (1) use of high-throughput screening for proteins after injury may provide a timecourse of how large numbers of critical events are simultaneously altered after CNS injury; (2) use of proteomic techniques could help to develop reliable biomarkers for CNS trauma and post-injury outcome; and (3) proteomic techniques could be useful for screening potential therapeutic targets.

Both the field of proteomics and the application of proteomic technologies to CNS trauma are in early stages.

Proteomics as a field is growing and changing almost daily. At this point, there does not appear to be a "magic bullet" proteomic technology that will be applicable for every research question. Each investigator will need to carefully consider the goals of the proposed experiments in determining the appropriate proteomic approach to utilize. There will be many technical challenges that CNS trauma investigators will face as the field advances. As they begin to utilize proteomics more regularly, the potential "payoff" to be attained with this type of data far outweighs the current difficulties and limitations.

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Accumulation of calpain and caspase-3 proteolytic fragments of brain-derived alphaII-spectrin in cerebral spinal fluid after middle cerebral artery occlusion in rats.

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Preclinical studies have identified numerous neuroprotective drugs that attenuate brain damage and improve functional outcome after cerebral ischemia. Despite this success in animal models, neuroprotective therapies in the clinical setting have been unsuccessful. Identification of biochemical markers common to preclinical and clinical cerebral ischemia will provide a more sensitive and objective measure of injury severity and outcome to facilitate clinical management and treatment. However, there are currently nc effective biomarkers available for assessment of stroke. Nonerythroid alphaI spectrin is a cytoskeletal protein that is cleaved by calpain and caspase-3 proteases to signature alphaII-spectrin breakdown products (alphaII-SBDPs) after cerebral ischemia in rodents. This investigation examined accumulation of calpain- and caspase-3-cleaved alphaII-SBDPs in cerebrospinal fluid (CSI of rodents subjected to 2 hours of transient focal cerebral ischemia produced by middle cerebral artery occlusion (MCAO) followed by reperfusion. After MCAO injury, full-length alphaII-spectrin protein was decreased in brain tissue and increased in CSF from 24 to 72 hours after injury. Whereas alphaI SBDPs were undetectable in sham-injured control animals, calpain but not caspase-3 specific alphaII-SBDPs were significantly increased in CSF after injury. However, caspase-3 alphaII-SBDPS were observed in CSF of some injured animals. These results indicate that alphaII-SBDPs detected in CSF after injury, particularly those mediated by calpain, may be useful diagnostic indicators of cerebral infarction that can provide important information abou specific neurochemical events that have occurred in the brain after acute stroke.

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ACCUMULATION OF CALPAIN AND CASPASE-3 PROTEOLYTIC FRAGMENTS OF BRAIN-DERIVED αII-SPECTRIN IN CSF AFTER MIDDLE CEREBRAL ARTERY OCCLUSION IN RATS

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Abstract

Preclinical studies have identified numerous neuroprotective drugs that attenuate brain damage and functional outcome after cerebral ischemia. Despite this success in animal models, neuroprotective therapies in the clinical setting have been unsuccessful. Identification of biochemical markers common to preclinical and clinical cerebral ischemia will provide a more sensitive and objective measure of injury severity and outcome to facilitate clinical management and treatment. However, there are currently no effective biomarkers available for assessment of stroke. Non-erythroid αII-spectrin is a cytoskeletal protein that is cleaved by calpain and caspase-3 proteases to signature αII-spectrin breakdown products (αII-SBDPs) after cerebral ischemia in rodents. This investigation examined accumulation of calpain- and caspase-3-cleaved αII-SBDPs in CSF of rodents subjected to 2 hours of transient focal cerebral ischemia produced by middle cerebral artery occlusion (MCAO) followed by reperfusion. Following MCAO injury, full-length αII-spectrin protein was decreased in brain tissue and increased in CSF from 24 hours to 72 hours after injury. Calpain- and caspase-3-specific αII-SBDPs were also increased in brain and CSF after injury. Levels of calpain-specific αII-SBDPs were greater at each post-injury time point than levels of caspase-3-specific αII-SBDPs. Levels of these proteins were undetectable in CSF of uninjured control rats. These results indicate that calpain- and caspase-3-cleaved αII-SBDPs in CSF may be useful diagnostic indicators of cerebral infarction that can provide important information about specific neurochemical events that have occurred in the brain after acute stroke.

Keywords: calpain, caspase-3, ischemia, stroke, biomarker, fodrin, α-spectrin, cerebrospinal fluid

Running title: Biomarkers of Stroke

Introduction

Acute ischemic stroke is a significant international health concern representing a potentially catastrophic debilitating medical emergency with poor prognosis for long-term disability. With the exception of diuretics, supportive measures, and when appropriate, thrombolytic therapy with recombinant tissue plasminogen activator (tPA), there are currently no approved drug treatments for ischemic brain injury (Grotta, 2002; Lees, 2002; Broderick and Hacke, 2002). Although a number of neuroprotective drugs have proven effective in reducing infarct size or improving functional outcome in preclinical testing, none have proven successful in clinical trials (Gladstone et al., 2002; Kidwell et al., 2001). Differences between preclinical and clinical trial outcome with neuroprotective drugs in acute ischemic stroke may be due to a variety of pitfalls that arise when attempting to extrapolate from animal to human investigations. These pitfalls may include differences in drug concentration and duration, differences in the window for therapeutic efficacy, differences in preclinical vs. clinical trial design, and the lack of standardized and sensitive outcome measures (Gladstone, et al., 2002; STAIR, 1999). For example, preclinical studies (typically in rodents) have traditionally utilized reduction of acute infarct volume as the primary measure of treatment efficacy, while clinical trials typically gauge treatment efficacy based on neurological and/or functional outcome (Gladstone et al., 2002). One approach to address these discrepancies in outcome measures is for preclinical and clinical trial designs to use outcome measures that are common to both human acute ischemic stroke and to preclinical animal models of ischemia. The use of common biochemical markers may provide such an approach.

Unlike other organ-based diseases where rapid diagnosis employing biomarkers (usually involving blood tests) prove invaluable to guide treatment of the disease, no such rapid and definitive diagnostic tests exist for acute ischemic brain injury. Biomarkers would have important applications in diagnosis, prognosis, and clinical research of ischemic brain injuries.

Simple and rapid diagnostic tools will immensely facilitate allocation of the major medical resources required to treat acute ischemic brain injuries. Accurate diagnosis in acute care environments can significantly enhance decisions about patient management, including decisions whether to admit or discharge patients or to administer other time consuming and expensive tests, including computed tomography (CT) and magnetic resonance imaging (MRI) scans. Biomarkers could provide major opportunities for the conduct of clinical research including confirmation of injury mechanism(s) and drug target identification. The temporal profile of changes in biomarkers could guide timing of treatment. Finally, biomarkers could provide a robust and sensitive clinical trial outcome measure that is obtainable more readily and with less expense than conventional neurological assessments, thereby significantly reducing the risks and costs of human clinical trials.

Previously reported biomarkers of cerebral ischemia include neuron-specific enolase (NSE), brain specific creatine kinase enzyme (CPK-BB), S-100β, and inflammatory cytokines such as IL-6 (Laskowitz et al., 1998). Of these, NSE and S-100β have been the most studied. After cardiac arrest, NSE elevations in serum and CSF have been correlated with neurological recovery (Roine et al., 1989; Martens, 1996; Dauberschmidt et al., 1991). Serum and CSF NSE values are reported to be elevated in rodent models of focal ischemia in proportion to the eventual infarct volume (Cunningham et al., 1991, 1996; Horn et al., 1995). In clinical trials, peak serum NSE values also predicted infarct volumes as shown by CT. However, correlating serum NSE values with functional outcome was less successful (Cunningham et al., 1991, 1996; Missler et al., 1997). S-100β protein has been studied most extensively for characterization of ischemic injuries after cardiac surgery and several reports have documented post-operative serum elevations (Sellman et al., 1992; Westaby et al., 1996). However, many of these reports do not include careful studies of neurological outcome and several investigators have recently criticized the diagnostic utility of S-100β during cardiac surgery (Anderson et al.,

2001). Thus, there is clearly a need for development of better biochemical markers for use in evaluating ischemic brain injury.

Our research efforts to develop biomarkers for traumatic brain injury (TBI) and acute ischemic brain injury have focused on αII-spectrin metabolic products as prototypical biochemical markers (Pike et al., 2001; Ringger et al., 2002). αII-spectrin is the major structural component of the cortical membrane cytoskeleton and is particularly abundant in axons and presynaptic terminals (Goodman et al., 1995; Riederer et al., 1986). Importantly, αII-spectrin is a major substrate for both and calpain and caspase-3 cysteine proteases (see **Fig. 1**), and the major calpain and caspase-3 cleavage sites of αII-spectrin have been well documented (Harris et al., 1988; Wang et al., 1998). Our laboratory has provided considerable evidence that αII-spectrin is processed by calpains and/or caspase-3 to signature cleavage products *in vivo* after TBI (Beer et al., 2000; Newcomb et al., 1997; Pike et al., 1998a, 2001) and in *in vitro* models of mechanical stretch injury (Pike et al., 2000), necrotic cell death (Zhao et al., 1999), apoptotic cell death (Pike et al., 1998b), and oxygen-glucose deprivation (Newcomb-Fernandez et al., 2001). Calpain and caspase-3 proteases also cleave αII-spectrin to signature proteolytic fragments in the brain in a rodent model of transient forebrain ischemia (Zhang et al., 2002). Although we have generated considerable laboratory data on the utility of αII-spectrin degradation as a biomarker for TBI in rodents (Pike et al., 2001), and more recently with preliminary data in human TBI patients (d'Avella et al., 2002), the present investigation is the first to provide evidence that calpain- and caspase-3-mediated αII-spectrin breakdown products (αII-SBDPs) can be detected in CSF after ischemic-reperfusion brain injury, and can used as biochemical markers in a rodent model of transient focal stroke in rats.

Methods

Surgical Procedures and Middle Cerebral Artery Occlusion: A "noninvasive" filament method of MCAO occlusion used extensively by our laboratories (Berti et al., 2002; Williams et

al., 2003) was used to produce cerebral ischemia in rats. The method described by Longa et al. (1989) and later modified in our laboratory by Britton et al. (1997) consists of blocking blood flow into the MCA with an intraluminal 3-0 monofilament nylon sterile suture with rounded tip introduced through an incision in the external carotid artery (ECA).

Under halothane anesthesia (5% halothane via induction chamber followed by 2% halothane via nose cone), the common carotid artery (CCA) was exposed at the level of external and internal carotid artery bifurcation with a midline neck incision. The internal carotid artery (ICA) was followed rostrally to the pterygopalatine branch and the ECA was ligated and cut at its lingual and maxillary branches. To prevent bleeding during suture insertion, the CCA and ICA were temporarily clamped with micro-aneurysm clips. The nylon suture was then introduced into the ICA via an incision on the ECA stump (the path of the suture can be monitored visually through the vessel wall) and advanced through the carotid canal approximately 20 mm from the carotid bifurcation until it becomes lodged in the narrowing of the anterior cerebral artery blocking the origin of the MCA. The skin incision was then closed using sterile autoclips. The endovascular suture remained in place for 2 hr at which time the rat was briefly re-anesthetized and the suture filament was retracted to allow reperfusion. For sham MCAO surgeries, the same procedure was followed but the filament was advanced only 10 mm beyond the internal-external carotid bifurcation and was left in place until sacrifice. During all surgical procedures, animals were maintained at 37.0°C by a homeothermic heating blanket (Harvard Apparatus, Holliston, MA).

Following surgery animals were placed in recovery cages with air temperature maintained at 22°C. During the 2 hr ischemia period and the initial 4 hr post-reperfusion period, 75-watt warming lamps were positioned directly over the top of each cage in order assist in maintaining normothermic body temperature throughout the experiment. Importantly, at the conclusion of each experiment, rat brains showing pathological evidence of subarachnoid hemorrhage upon

necropsy were excluded from the study. Also, all rats exhibiting convulsant behaviors at any time post MCAO were excluded from the experiment, as well as those animals not showing maximal neurologic impairment (NS=10, see description below) immediately prior to the 2 hr reperfusion.

Brain Tissue and CSF Collection: Brain (cortex and hippocampus) and CSF was collected from animals at various intervals after sham-injury or MCAO as previously described by our laboratory (Pike et al., 2001). At the appropriate time-points, MCAO or sham-injured animals were anesthetized as described above and secured in a stereotactic frame with the head allowed to move freely along the longitudinal axis. The head was flexed so that the external occipital protuberance in the neck was prominent and a dorsal midline incision was made over the cervical vertebrae and occiput. The atlanto-occipital membrane was exposed by blunt dissection and a 25G needle attached to polyethylene tubing was carefully lowered into the cisterna magna. Approximately 0.1 to 0.15 ml of CSF was collected from each rat. Following CSF collection, animals were removed from the stereotactic frame and immediately killed by decapitation. Ipsilateral and contralateral (to the site of infarct) cortices were then rapidly dissected, rinsed in ice cold PBS, and snap frozen in liquid nitrogen. Cortices were excised to the level of the white matter and extended ~4 mm laterally and ~7 mm rostrocaudally. CSF samples were centrifuged at 4000 g for 4 min. at 4°C to clear any contaminating erythrocytes. Cleared CSF and frozen tissue samples were stored at -80°C until ready for use. Cortices were homogenized in a glass tube with a Teflon dounce pestle in 15 volumes of an ice-cold triple detergent lysis buffer (20 mM Hepes, 1 mM EDTA, 2 mM EGTA, 150 mM NaCl, 0.1% SDS, 1.0% IGEPAL 40, 0.5% deoxycholic acid, pH 7.5) containing a broad range protease inhibitor cocktail (Roche Molecular Biochemicals, cat. #1-836-145).

Immunoblot Analyses of CSF and Cortical Tissues: Protein concentrations of tissue homogenates and CSF were determined by bicinchoninic acid microprotein assays (Pierce Inc.,

Rockford, IL) with albumin standards. Protein balanced samples were prepared for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in twofold loading buffer containing 0.25 M Tris (pH 6.8), 0.2 M DTT, 8% SDS, 0.02% bromophenol blue, and 20% glycerol in distilled H_2O. Samples were heated for 10 min. at 100°C and centrifuged for 1 min. at 10,000 rpm in a microcentrifuge at ambient temperature. Twenty micrograms of protein per lane was routinely resolved by SDS-PAGE on 6.5% Tris/glycine gels for 1 hour at 200 V. Following electrophoresis, separated proteins were laterally transferred to polyvinylidene fluoride (PVDF) membranes in a transfer buffer containing 0.192 M glycine and 0.025 M Tris (pH 8.3) with 10% methanol at a constant voltage of 100 V for 1 hour at 4°C. Blots were blocked for 1 hour at ambient temperature in 5% nonfat milk in TBS and 0.05% Tween-20. Panceau Red (Sigma, St. Louis, MO) was used to stain membranes to confirm successful transfer of protein and to insure that an equal amount of protein was loaded in each lane.

Antibodies and Immunolabeling of PVDF Membranes: Immunoblots containing brain or CSF protein were probed with an anti-αII-spectrin (fodrin) monoclonal antibody (FG 6090 Ab; clone AA6; cat. # FG 6090; Affiniti Research Products Limited, UK) that detects intact non erythroid αII-spectrin (280 kDa) and 150, 145, and 120 kDa cleavage fragments to αII-spectrin. A cleavage product of 150 kDa is initially produced by calpains or caspase-3 proteases (each proteolytic cleavage yields a unique amino-terminal region; Nath et al., 1996; Wang et al., 1998; **Fig. 1**). The calpain-generated 150 kDa product is further cleaved by calpain to yield a specific calpain signature product of 145 kDa (Harris et al., 1988; Nath et al., 1996) whereas the caspase-3 generated 150 kDa product is further cleaved by caspase-3 to yield an apoptotic-specific caspase-3 signature product of 120 kDa (Nath et al., 1998; Wang et al., 1998; Wang, 2000). Following an overnight incubation at 4°C with the primary antibody (FG 6090 Ab, 1:4000 for brain tissue and 1:2000 for CSF), blots were incubated for 1 hr at ambient temperature in 3% nonfat milk that contained a horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000

dilution). Enhanced chemiluminescence (ECL, Amersham) reagents were used to visualize immunolabeling on Kodak Biomax ML chemiluminescent film.

Statistical Analyses: Semi-quantitative evaluation of protein levels detected by immunoblotting was performed by computer-assisted densitometric scanning (AlphaImager 2000 Digital Imaging System, San Leandro, CA). Data were acquired as integrated densitometric values and transformed to percentages of the densitometric levels obtained on scans from sham-injured animals visualized on the same blot. Data was evaluated by least squares linear regression followed by ANOVA. All values are given as mean ± SEM. Differences were considered significant if $p < 0.05$.

Results

Proteolysis of αII-Spectrin in the Ipsilateral Cortex by Calpains and Caspase-3 After MCAO Injury. In the ipsilateral cortex, MCAO injury caused significant ($p < 0.05$) accumulation of the non-specific 150 kDa αII-SBDP (generated by calpain and/or caspase-3), and of the calpain-specific 145 kDa αII-SBDP at all post-injury time points as compared to sham-injured control rats (**Fig. 2**). Levels of the calpain-specific 145 kDa αII-SBDP were 304%, 282%, and 301% of sham-injured control values at 24, 48, and 72 hours post-injury, respectively (**Fig. 3**). Levels of the non-specific 150 kDa αII-SBDP closely matched levels of the 145 kDa fragment, and were 276%, 275%, 268% of sham-injured control valules at 24, 48, and 72 hours, respectively (**Fig. 3**). MCAO injury also resulted in more modest, but significant ($p < 0.05$ to $p < 0.001$) levels of caspase-3-specific 120 kDa αII-SBDPs at all post-injury time points (**Fig. 2**). Levels of the caspase-3-specific 120 kDa fragment were 131%, 132%, and 140% of sham-injured control values at 24, 48, and 72 hours post-injury, respectively (**Fig. 3**). Although levels of the caspase-3-specific 120 kDa αII-SBDP were smaller than those produced by calpains,

between animal variability was much lower for levels of caspse-3 αII-SBDPs compared to the variability for levels of calpain αII-SBDPs.

In the contralateral cortex, MCAO injury caused no significant accumulation of calpain- or caspase-3-specific αII-SBDPs at any post-injury time point as compared to sham-injured control rats (**Fig. 2**).

Accumulation of Calpain and Caspase-3 Mediated αII-SBDPs in CSF after MCAO Injury. Cerebrospinal fluid levels of αII-spectrin and αII-SBDPs were undetectable in sham-injured control animals (**Fig. 2**). However, after MCAO injury accumulation of full length αII-spectrin (280 kDa) and the 150 kDa, 145 kDa, and 120 kDa αII-SBDPs were overtly apparent on immunoblots at various post-injury time points (**Fig. 2**). Levels of the full length αII-spectrin protein were increased in CSF of MCAO injured animals and were 144%, 453%, and 395% of sham-injured control levels at 24, 48, and 72 hours post-injury. However, there was considerable between animal variability in levels of the full length protein; thus, quantitative analysis failed to reach statistical significance (**Fig. 3**). Levels of the non-specific 150 kDa αII-SBDP, and of the calpain-specific 145 kDa αII-SBDP, were apparent on immunoblot at all post-injury time points, but only levels at 48 hours and 72 hours post-injury reached statistical significance ($p < 0.001$). Levels of the non-specific 150 kDa αII-SBDP were 216%, 523%, and 467% of sham-injured control animals, and levels of the calpain-specific 145 kDa αII-SBDP were 268%, 626%, and 546% of sham-injured control values at 24, 48, and 72 hours post-injury, respectively (**Fig. 3**). Levels of the caspase-3-specific 120 kDa αII-SBDP were 84%, 439%, and 110% of sham-injured control levels at 24, 48, and 72 hours post-injury, respectively (**Fig. 3**). Although levels of caspase-3-specific 120 kDa αII-SBDPs were 439% of sham-injured control values at 48 hours post-injury, large between animal variability precluded statistical significance.

Linear Regression Analyses of Cortical versus CSF Levels of αII-Spectrin and αII-SBDPs. Least squares linear regression was calculated to examine the relationship between cortical and CSF levels of αII-spectrin and αII-SBDPs over days post-injury in sham-control and MCAO-injured animals. The slopes of the regression lines for brain and CSF protein levels were analyzed by ANOVA.

The slope of the regression line for protein levels of the full-length 280 kDa αII-spectrin in the cortex across days post-injury was only slightly negative (m = -4.872), indicating modest decreases in total αII-spectrin protein (**Fig. 4**). The slope of the regression line for CSF levels of the full-length αII-spectrin protein across days post-injury was positive (m = 113.5), indicating increased accumulation of αII-spectrin in CSF from 24 h to 72 h after MCAO injury.

Slopes of the regression lines for the 150 kDa αII-SBDP in cortex and CSF across days post-injury were both positive (m = 53.59 and m = 145.4, respectively; **Fig. 4**). This result is consistent with the immunoblot data demonstrating increased accumulation of the 150 kDa αII-SBDP in cortex and CSF after MCAO injury. ANOVA indicated no significant difference (F = 2.14, p = 0.2172) between cortical and CSF slopes. This result indicates that rate of accumulation of the non-specific 150 kDa αII-SBDP in brain and CSF over days post-injury was approximately equivalent.

Slopes of the regression line for the 145 kDa αII-SBDP in cortex and CSF across days post-injury were both positive (m = 60.63 and m = 170.5, respectively; **Fig. 4**). This result is also consistent with the immunoblot data demonstrating increased accumulation of the 145 kDa αII-SBDP in cortex and CSF after MCAO injury. ANOVA indicated no significant difference (F = 2.50, p = 0.1889) between cortical and CSF slopes. This result indicates that rate of

accumulation of the calpain-specific 145 kDa αII-SBDP in brain and CSF over days post-injury was approximately equivalent.

Slopes of the regression line for the 120 kDa αII-SBDP in cortex and CSF across days post-injury were both slightly positive (m = 15.12 and m = 41.28, respectively; **Fig. 4**). This result is consistent with the immunoblot data demonstrating relatively small increases in accumulation of the 120 kDa αII-SBDP in cortex and CSF after MCAO injury. Again, ANOVA indicated no significant difference (F =0.08, p = 0.7861) between cortical and CSF slopes, indicating that rate of accumulation of the caspase-3 specific 120 kDa αII-SBDP in brain and CSF over days post-injury was approximately equivalent.

Discussion

This paper provides further evidence supporting the use of calpain and caspsase-3 specific αII-SBDPs as surrogate neurochemical markers of CNS injury. Previous data from our laboratory demonstrate that TBI causes robust and detectable accumulation of calpain-mediated αII-SBDPs (and to a lesser extent, caspase-3-mediated αII-SBDPs) in CSF of brain injured rodents (Pike et al., 2001). We now demonstrate that a rodent stroke model of focal ischemic injury also results in increased levels of calpain and caspase-3 αII-SBDPs in post-injury CSF. The results of these two studies are important in that they provide the first evidence that extra-parenchymal detection of specific protein metabolic products can be used as unequivocal biochemical markers for specific neurochemical events (i.e., calpain and caspase-3 activation) that have occurred in the injured brain in at least two preclinical models of brain injury (traumatic and ischemic). Importantly, recent preliminary clinical studies in patients with severe TBI also indicate robust levels of calpain and caspase-3 mediated αII-SBDPs in CSF (d'Avella et al., 2002).

Analysis of specific biochemical markers is a mandatory component of diagnosing dysfunction in a number of organs, including the use of troponin assays in patients with acute coronary syndromes (Newby et al., 2003). Indeed, troponin testing has rapidly evolved from its initial role in aiding diagnosis of myocardial infarction to a more complex role for risk stratification and guidance of treatment strategies (Newby et al., 2003). However, there are no such biomarkers of proven clinical utility for TBI and cerebral ischemia. In the case of TBI, this may be due, in part, to the fact that TBI is difficult to assess and clinical examinations are of restricted value during the first hours and days after injury. For instance, conventional diagnoses of TBI are based on neuroimaging techniques such as CT scanning, MRI, and single-photon emission CT scanning (Jacobs et al., 1996; Kant et al., 1997; Mitchener et al., 1997). CT scanning has low sensitivity to diffuse brain damage and the availability of MRI is limited (Kesler et al., 2000; Levi et al., 1900). In addition, single-photon emission CT scanning detects regional blood-flow abnormalities not necessarily related to structural damage. In the case of stroke, investigators have also generally recognized the need for more objective assessments of outcome, including the use of biochemical markers (Dirnagl et al., 1999; Zaremba et al., 2001). The approval of tPA as a treatment for acute stroke has additionally highlighted the potential utility of biochemical markers. For example, diagnosis of stroke is relatively straightforward when patients present with typical symptoms; however, often symptoms of stroke are more subtle and can delay diagnosis by hours or days (Elkind, 2003). Additionally, other causes of neurological symptoms, such as seizure, migraine, vasospasm, syncope, and peripheral vestibulopathy, can be indistinguishable from symptoms of thromboembolic transient ischemic attacks (Johnston et al., 2003). Thus, a rapid and reliable biochemical marker of stroke will facilitate diagnosis and might give assurance to physicians considering administering thrombolytic agents for treatment of acute ischemic stroke.

Our laboratories' assessment of αII-SBDPs as biochemical markers in models of TBI and focal cerebral ischemia may result in considerable improvement over currently existing biochemical markers of CNS injury. For instance, other putative biomarkers of CNS injury (e.g., CPK-BB, NSE, S-100β, lactate dehydrogenase, etc.) are of limited value due to a lack of specificity to CNS tissues, unreliability in predicting outcome, and because they provide no specific information regarding neurochemical pathology of injured CNS tissue. Recent studies have also examined the utility of cleaved tau protein (cτP) as a predictor of outcome. However, while cτP is axonal specific, it also provides no information about specific neurochemical events that have occurred in the injured CNS. Furthermore, recent studies have presented conflicting evidence as to the utility of cτP as a predictor of outcome after TBI in humans (Chatfield et al., 2002; Zemlan et al., 1999). In contrast, αII-SBDPs offer several advantages as compared to the putative biomarkers just described. For instance, αII-SBDPs provide concurrent information on post-injury activity of two important proteolytic enzymes (calpain and caspase-3). Low basal levels of these proteases further optimizes their utility as markers of cell injury. Another important characteristic is that αII-spectrin protein is not localized in erythrocytes (Goodman et al., 1995; Riederer, et al., 1986). Blood is a major source of CSF contamination after TBI and hemorrhagic ischemia. Results from our previously published studies in TBI clearly demonstrate that αII-spectrin and αII-SBDPs are not detectable in whole blood samples. In contrast, the erythroid isoform of spectrin, αI-spectrin, is detectable in both blood and brain tissues (Pike et al., 2001).

However, one disadvantage is that while αII-spectrin is highly enriched in brain, it is not specific to brain tissue. While this is not a concern for CSF detection of αII-SBDPs, it could be problematic for detection of αII-SBDPs in serum as human head-injured patients often present with multi-organ trauma. Additional studies in preclinical models and in human patients are needed to clarify this issue.

An ideal biomarker for a particular neurological disease is one that is 100% specific and sensitive for that particular disease. However, with TBI or stroke, it is not critical that a biomarker be specific to one or the other disorder, rather, the biomarker need only indicate, with as much sensitivity as possible, the severity of brain damage that has occurred as a result of brain trauma or cerebral infarction (although a biomarker that can rapidly and accurately discriminate between hemorrhagic and thrombolytic stroke would certainly be useful). The use of calpain- and caspase-3-mediated αII-SBDPs could provide a powerful approach for determining the severity of brain damage caused by a TBI or stroke, and could also provide a clinical tool for monitoring the duration of the acute injury response and the effects of emergency or therapeutic interventions. For instance, calpain and caspase-3 are potent mediators of cell death that can be rapidly activated in response to traumatic (Beer et al., 2000; Pike et al., 1998a; Sullivan et al., 2002) or ischemic brain injury (Davoli et al., 2002; Zhang et al., 2002), and brain regions with the highest accumulation of αII-SBDPs have the highest level of neuronal cell death (Roberts-Lewis et al., 1994; Newcomb et al., 1997). Importantly, calpain and caspsase-3 can be concurrently or independently activated after TBI (Pike et al., 1998a) or cerebral ischemia (Zhang et al., 2002), and the temporal duration of activity can vary for each protease. Thus, the ability to monitor both calpain and caspase-3 activation during the acute period of CNS injury is a major advantage of αII-SBDPs over other biomarkers. Indeed, recent preliminary data obtained from CSF of severely injured TBI patients indicate that temporal accumulation of calpain- and caspase-3-mediated αII-SBDPs show different patterns of temporal expression that vary in each patient (d'Avella et al., 2002). This result is similar to our preclinical TBI and ischemic injury models in which accumulation of calpain and/or caspase-3 αII-SBDPs also varies between individual animals. This variability emphasizes the heterogeneous nature of TBI and ischemic pathology, and points to important implications for

individualized treatment of human brain injured patients that is tailored to specific neurochemical cascades operative in the injured brain.

In summary, this paper provides further evidence supporting the use of calpain- and caspase-3-mediated αII-SBDPs as neurochemical markers of CNS injury. Although numerous other proteins, peptides, amino acids, etc., have been identified in CSF after TBI and acute cerebral ischemia, no such surrogate marker of CNS injury has yet provided a window of insight into specific neurochemical events that have occurred as a result of traumatic or ischemic brain injury. The use of protease-specific αII-SBDPs as biomarkers offers several advantages over existing biomarkers of traumatic or ischemic brain injury, including the ability to provide concurrent information about the activity of two major proteolytic effectors of cell death. Additional studies to further characterize the sensitivity of αII-SBDPs (e.g., in serum and across injury magnitudes) are ongoing. In addition, it is thought that the development of other CNS-specific biomarkers used in conjunction with αII-SBDPs will provide researchers and clinicians with powerful tools for diagnosing and assessing CNS injury, for monitoring recovery, and for guiding appropriate administration of therapeutic compounds. Finally, it is thought that recent advancements in antibody-based specific identification technologies will facilitate development of rapid, sensitive, and easy-to-use kits for research and clinical environments.

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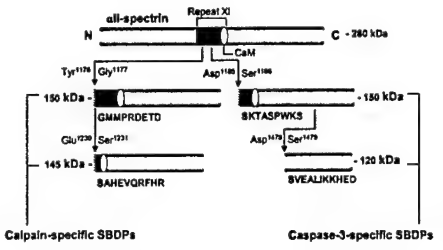
Figure Legends

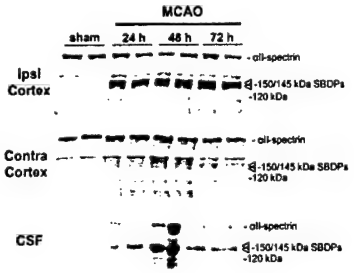
Figure 1. Calpain and caspase-3 cleavage of non-erythroid αII-spectrin to protease-specific αII-spectrin breakdown products (SBDPs) . Illustrated are the calpain cleavages (left) in αII-spectrin that result in calpain-specific SBDPs (150 and 145 kDa) and the caspase-3 cleavages (right) in αII-spectrin that result in caspase-3-specific αII-SBDPS (150 and 120 kDa). Both proteases cleave αII-spectrin in repeat 11 near the calmodulin binding domain (CaM) to produce 150 kDa αII-SBDPs with unique N-terminal regions. A second cleavage by calpain in repeat 11 results in a calpain-specific 145 kDa αII-SBDP, while caspase-3 cleaves the protein in repeat 13 to produce a unique, apoptotic-specific 120 kDa fragment. For definitively identified cleavages, the flanking amino acids and initial N-terminal amino acid sequence is given.

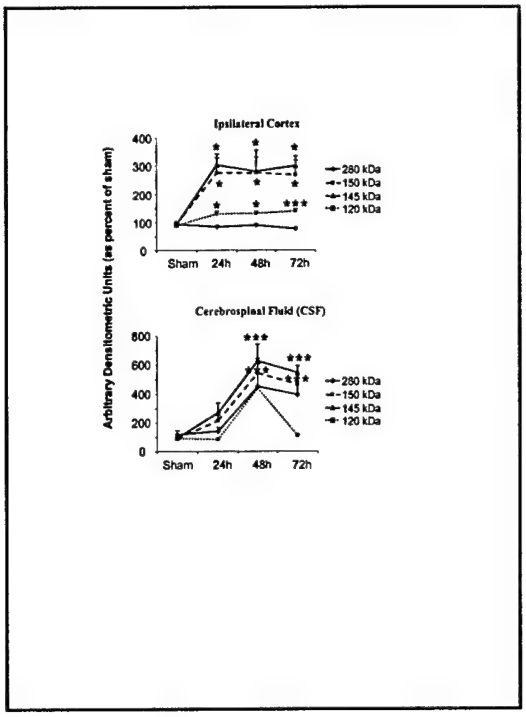
Figure 2. MCAO injury causes accumulation of full-length αII-spectrin (280 kDa) protein, and calpain-mediated 145 kDa and caspase-3 mediated 120 kDa αII-SBDPs in CSF. MCAO resulted in proteolysis of constitutively expressed brain αII-spectrin (280 kDa) in ipsilateral but not contralateral cortex. The caspase-3-mediated, apoptotic-specific 120 kDa αII-SBDP was also increased in ipsilateral cortex after ischemia compared to sham-injured controls. Marked increases in the calpain-specific 145 kDa αII-SBDP were detected in brain and CSF of MCAO animals, but not in sham-injured animals, at all time points. Interestingly, while increased levels of the caspase-3-specific 120 kDa αII-SBDP were detected at all post-injury time points in the ipsilateral cortex, CSF levels were only detected at 48 hours post-injury.

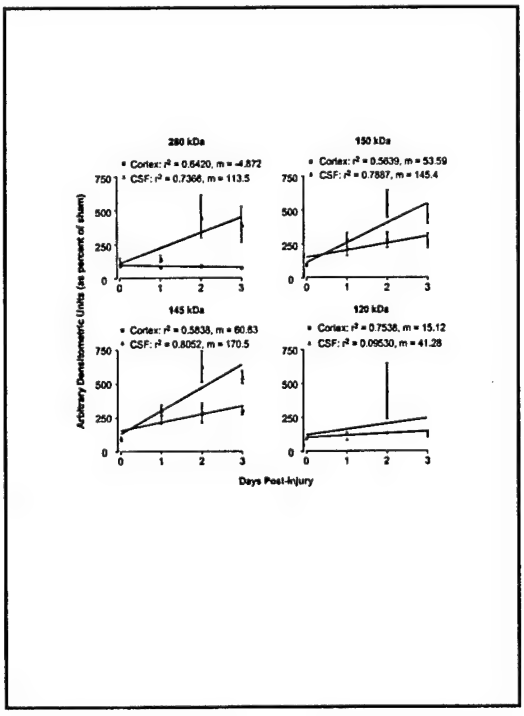
Figure 3. Mean (± s.d.) arbitrary densitometric units obtained from full-length 280 kDa αII-spectrin protein and the 150 kDa, 145 kDa, and 120 kDa αII-SBDPs. Densitometric units were converted to percent of sham-injured values. Decreases in 280 kDa αII-spectrin and increases in 150 kDa, 145 kDa, and 120 kDa αII-SBDPs (ipsilateral cortex) were associated with concomitant increases of these proteins in the CSF. Note that while 150 kDa and 145 kDa SBDPs were visibly detectable in CSF on western blot at 24 hours post-injury, densitometric levels were not statistically significant due to greater variability at this time point. Similarly, although the full-length 280 kDa spectrin protein and the 120 kDa αII-SBDPs were visibly detectable in CSF, large variability in protein levels between animals resulted in inability to detect statistical significance. *$p<0.05$ and ***$p<0.001$.

Figure 4. Mean (± s.d.) cortical vs. CSF levels of αII-spectrin (280 kDa) and αII-SBDPs (150, 145, and 120 kDa) over days post-injury. Least squares regression lines of brain and CSF spectrin and SBDP levels were plotted on the same graph. Pearson correlation coefficients for each regression line are indicated. Results indicate that parenchymal decreases in levels of native αII-spectrin (280 kDa) are associated with increases in CSF accumulation while increased parenchymal levels of calpain-mediated αII-SBDPs (150 & 145 kDa) are associated with increased CSF accumulation. On average, there were no changes in parenchymal or CSF levels of the caspase-3-mediated 120 kDa αII-SBDP across days. However, individual rats at different time points (particularly 48 hours post-injury) showed some increase in CSF levels of the 120 kDa product.


αII-spectrin
Repeat XI
N
C - 280 kDa
CaM
Tyr1176 Gly1177
Asp1185 Ser1186
150 kDa -
GMMPRDETD
- 150 kDa
SKTASPWKS
Glu1230 Ser1231
Asp1478 Ser1479
145 kDa -
SAHEVQRFHR
- 120 kDa
SVEALIKKHED
Calpain-specific SBDPs
Caspase-3-specific SBDPs


MCAO
sham
24 h
48 h
72 h
Ipsi Cortex
- αII-spectrin
-150/145 kDa SBDPs
-120 kDa
Contra Cortex
- αII-spectrin
-150/145 kDa SBDPs
-120 kDa
CSF
- αII-spectrin
-150/145 kDa SBDPs
-120 kDa


Ipsilateral Cortex
Arbitrary Densitometric Units (as percent of sham)
400
300
200
100
0
Sham
24h
48h
72h
280 kDa
150 kDa
145 kDa
120 kDa
Cerebrospinal Fluid (CSF)
800
600
400
200
0
Sham
24h
48h
72h
280 kDa
150 kDa
145 kDa
120 kDa


280 kDa
Cortex: r² = 0.6420, m = -4.872
CSF: r² = 0.7366, m = 113.5
150 kDa
Cortex: r² = 0.5639, m = 53.59
CSF: r² = 0.7887, m = 145.4
145 kDa
Cortex: r² = 0.5838, m = 60.83
CSF: r² = 0.8052, m = 170.5
120 kDa
Cortex: r² = 0.7536, m = 15.12
CSF: r² = 0.09530, m = 41.28
Arbitrary Densitometric Units (as percent of sham)
750
500
250
0
0
1
2
3
Days Post-Injury

Biomarkers Of Proteolytic Damage Following Traumatic Brain Injury

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Key words: biomarkers, surrogate markers, protease, caspase, calpain, traumatic brain injury

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Biomarkers Of Proteolytic Damage Following Traumatic Brain Inury

Introduction

Brain injury resulting from traumatic, ischemic and/or chemical etiology is a significant international health concern, representing a potentially catastrophic debilitating medical emergency with poor prognosis for long-term disability. It represents a major problem to military care, accounting for 25% of all combat casualties and is the leading cause of death (approaching 50% incidence) among wounded soldiers reaching Echelon I medical treatment [8]. In civilian life, the incidence of brain injury and resultant long-term disabilities caused by traumatic insults (automobile accidents, gunshots, sports, etc.) and ischemic events (strokes, cerebral hemorrhage, cardiac arrest, etc.) are several orders of magnitude greater. There are more than 1 million traumatic brain injury (TBI) cases that are treated and released from an emergency department annually in the United States resulting in more than 230,000 hospitalizations, 50,000 deaths and 80,000 disabilities. Among all age groups, the top three causes of TBI are motor vehicle accidents, falls and violence [1] . Despite modern automobile design and injury prevention campaigns, important causes of TBI in children such as ejections from cars during traffic accidents, have increased in recent years [111]. The current estimation is 5.3 million Americans live with TBI-related disability. TBI is the greatest cause of death and disability in young people less than 24 years old [12].

With the exception of supportive measures, there are currently no approved drug treatments for TBI [77]. There have been a large number of clinical trials studying potential therapies for traumatic brain injury (TBI) that have resulted in negative findings with a cost of over $200 million [15, 30]. Many investigators have pointed out that the absence of biochemical markers of injury could have contributed to these failures [77, 104]. Unlike other organ-based diseases where rapid diagnosis employing biomarkers (usually involving blood tests) prove

invaluable to guide treatment of the disease, no such rapid, definitive diagnostic tests exist for TBI to provide physicians with quantifiable neurochemical markers to help determine the seriousness of the injury, the anatomical and cellular pathology of the injury, and to guide implementation of appropriate triage and medical management.

Criteria For Biochemical/Surrogate Markers

In the course of research on biomarkers, our laboratories have developed criteria for biomarker development. Useful biomarkers should employ readily accessible biological material such as CSF or blood (CSF is routinely accessible in severely injured TBI patients), predict the magnitude of injury and resulting functional deficits and possess high sensitivity and specificity, have a rapid appearance in blood and be released in a time-locked sequence after injury. Ideally, biomarkers should employ biological substrates unique to the CNS and provide information on injury mechanisms, a criterion often used to distinguish biochemical markers from surrogate markers of injury, which usually do not provide information on injury mechanisms. Potential gender and age related differences on biomarker profiles are also important and should be taken into account when developing useful biochemical markers [43].

Uses Of Biomarkers

Biomarkers would have important applications in diagnosis, prognosis and clinical research of brain injuries. Simple, rapid diagnostic tools will immensely facilitate allocation of the major medical resources required to treat TBI and other brain injuries. Accurate diagnosis in acute care environments can significantly enhance decisions about patient management including decisions whether to admit or discharge or administer other time consuming and expensive tests including computer tomography (CT) and magnetic resonance imaging (MRI) scans. Biomarkers

could have important prognostic functions especially in patients suffering mild TBI, which make up an estimated 80% of the 2.5 to 6.5 million individuals who suffer from lifelong impairment as a result of TBI [2, 83]. Accurate identification of these patients could facilitate development of guidelines for return to duty, work or sports activities and also provide opportunities for counseling of patients suffering from these deficits. Biomarkers could provide major opportunities for the conduct of clinical research including confirmation of injury mechanism(s) and drug target identification. The temporal profile of changes in biomarkers could guide timing of treatment and assist in monitoring the response to therapy and intervention. Finally, biomarkers could provide a clinical trial outcome measure obtainable much more cheaply and readily than conventional neurological assessments, thereby significantly reducing the risks and costs of human clinical trials. Relevant, easily available biomarkers are needed in order to maximize chances of success in developing long awaited effective drugs for traumatic brain injury [77].

Current Status of Research on Markers of Traumatic Brain Injury

Analysis of specific biochemical markers has provided useful information on the mechanism and diagnosis specific organ dysfunction in humans [112]. However, although analysis of cerebrospinal fluid, cerebral microdialysis samples, and brain tissue specimens has provided insight into the mechanisms of brain injury [61, 63], there are no biomarkers of proven clinical utility for TBI.

TBI is difficult to assess and clinical examinations are of restricted value during the first hours and days after injury. Conventional diagnoses of TBI are based on neuroimaging techniques such as CT scanning, MRI and single-photon emission CT scanning [46, 58, 72]. CT scanning has low sensitivity to diffuse brain damage, and the availability of MRI is limited [60,

64]. Single-photon emission CT scanning detects regional blood-flow abnormalities not necessarily related to structural damage.

A recent review of biomarkers of TBI highlighted the need for biomarker development [43]. The most studied potential biochemical markers for TBI include creatine kinase (CK), glial fibrillary acidic protein (GFAP), lactate dehydrogenase (LDH), myelin basic protein (MBP), neuron-specific enolase (NSE) and S-100 proteins. The bulk of research in TBI has focused on NSE and S-100β. The specificity of NSE for brain is high [49] , sex- and age-related variability is low [28, 51, 74, 86, 98, 120, 121, 133], and NSE is rapidly detectible in serum after TBI [129]. However, studies relating NSE serum levels to admission GCS in patients with severe TBI show conflicting results. Similar data have been reported concerning relationships with CT scan findings, ICP and long-term outcomes. In mild TBI, NSE failed to separate patients from controls [43, 44, 108, 131]. Thus, NSE is predominantly used as a marker for tumors [22]. NSE is also released in the blood by hemolysis, which could be a major source of error [22].

The S-100 protein family now consists of 19 members, of which S-100B is the one viewed as a marker of brain damage [49, 65], although it is present in other tissues such as adipocytes and chondrocytes [40]. Investigators have reported S-100β serum levels correlate to both GCS scores, neuroradiologic findings at admission and long-term outcomes [100, 99, 128]. However, investigators have recently raised questions about the utility of S-100β reporting that high serum levels of S-100β are detectible in trauma patients not having head injuries, a factor not adequately controlled for in earlier studies [3]. In addition, serum levels of S-100β following mild TBI do not show strong correlations with neuropsychological outcome [107]. Research in this area continues and recent reports have indicated the potential utility of measures of blood GFAP [71], spinal fluid Interleukin-6 [115] and cleaved tau protein in serum [45, 114] and spinal fluid [135] following brain injury.

Investigators have also generally recognized the need for more objective assessments of outcome following stroke, including biochemical markers [27, 68]. The approval of tPA as a treatment for acute stroke has additionally highlighted the potential utility of biochemical markers. Use of tPA may be hindered by diagnostic concerns because neurological deficits accompanying stroke can mimic those seen during transient ischemic attacks, complex migraine, space-occupying lesions and post-ictal paralysis. A reliable biochemical marker might give assurance to physicians considering administering thrombolitic agents for acute stroke [41, 50].

Previously reported biomarkers of cerebral ischemia include NSE, brain specific creatine kinase enzyme (CPK-BB), S-100β and inflammatory cytokines such as IL-6 [76, 89]. NSE and S-100β have been the most studied. After cardiac arrest, NSE elevations in serum and CSF have been correlated with neurological recovery [26, 67, 106]. Serum and CSF NSE values were reported to be elevated in rodent models of focal ischemia in proportion to the eventual infarct volume [24, 25, 42]. In clinical trials, peak serum NSE values also predicted infarct volumes as shown by CT. Correlating serum NSE values with functional outcome was less successful [24, 25, 70], possibly because functional neurological deficit is influenced as much by location of brain injury as by infarct size [70]. S-100β protein has been studied most extensively for characterization of ischemic injuries after cardiac surgery, and several reports have documented post-operative serum elevations [29, 113, 127]. However, many of these reports do not include careful studies of neurological outcome, and several investigators have recently criticized the diagnostic utility of S-100β during cardiac surgery. [3].

Proteolytic Damage and the Pathobiology of Traumatic Brain Injury

After TBI, brain cells can deteriorate following more than one pathway, and many genes and proteins may be involved. Programmed cell death is an evolutionarily conserved form of cell suicide that occurs widely throughout development [13]. This type of cell death often has the

morphological appearance of apoptosis [119]. Apoptosis occurs following TBI in animals [20, 59, 130] and humans [16, 17]. Studies of apoptosis pose special challenges since there are multiple apoptotic pathways, and apoptosis is extremely sensitive to a number of variables including injury type and magnitude [10, 14, 101], cell type [38, 57] and stimulation/antagonism of specific receptors [14, 19, 23, 38, 39, 48].

The molecular events occurring after TBI are just beginning to be understood. Elevated neuronal calcium levels activate a number of calcium-dependent enzymes such as phospholipases [84], kinases [132], phosphatases [75], and proteases [5, 81], all of which can modulate post-TBI cytoskeletal protein loss. Caspase-3 is a member of the caspase family of cysteine proteases. Activated caspase-3 has many cellular targets that, when severed and/or activated, produce the morphologic features of apoptosis [18]. Calpains are calcium-activated, neutral cysteine proteases with relative selectivity for proteolysis of a subset of cellular proteins. Calpain activation has been implicated in different models of apoptosis and in different cell types, including neurons [93]. Understanding of the contributions of calpains and caspases to cell injury/death following TBI may have important diagnostic and therapeutic implications.

Contributions of Caspase-3 and Calpain to Cell Death Following Traumatic Brain Injury

Numerous studies from our own [6, 91, 93] and other laboratories [31, 35, 73] have provided evidence that the caspase family of cysteine proteases is an important intracellular effector of apoptosis in various cell lines and apoptotic models. Caspase 3-like proteases have been shown to cleave a variety of cytoplasmic, nuclear and cytoskeletal proteins during apoptosis including αII-spectrin [69, 78, 79, 122], poly(ADP-ribose) polymerase (PARP: 51) and others (52-57). *In vitro* studies in our laboratories using a model of stretch injury have demonstrated caspase-3 processing of α-spectrin to the apoptotic-linked 120-kDa fragment 24 hours after moderate, but not mild or severe injury [6]. I*n vivo* studies have provided evidence of caspase-3

activation following TBI. First, Clark et al. demonstrated cleavage of capsase-3 to its p18 and p12 subunits in humans [17]. Yakovlev et al. reported that TBI increased caspase-3, but not caspase-1, activity [130]. Caspase-3 inhibition reduced DNA fragmentation and TUNEL staining and improved behavioral outcome. We have also concurrently examined caspase-3 and calpain activation after TBI. Distinct regional and temporal patterns of calpain/caspase-3 processing of αII-spectrin in brain regions ipsilateral to the site of injury after TBI have been observed. Caspase-3-mediated break down products (BDP's) to αII-spectrin were absent in the cortex but showed significant increases in hippocampus and striatum early (hours) after TBI [92]. Immunohistological examinations revealed increased expression of the proteolitically active subunit of caspase-3, p18, in neurons, astrocytes, and oligodendrocytes from 6 to 72 hours following controlled cortical impact injury. Moreover, concurrent assessment of nuclear histopathology using hematoxylin identified p18-immunopositive cells exhibiting apoptotic-like morphological profiles in the cortex ipsilateral to the injury site [6].

Calpains are Ca^{2+} activated cysteine proteases that have been implicated in a variety of neuropathological conditions [55, 126]. Intracellular substrates of activated calpain include cytoskeletal proteins, calmodulin-binding proteins, enzymes involved in signal transduction, membrane proteins and transcription factors [110, 118, 125]. While calpain activation has historically been associated with necrotic cell death [82], calpain activation has also been implicated in different models of apoptosis and in different cell types, including neurons [7, 52, 78, 117, 123]. Research in our own and other laboratories have documented calpain activation following TBI *in vivo* [55]. TBI results in altered Ca^{2+} homeostasis [134] and activates several Ca^{2+} -dependent enzymes including the calpains. Overactivation of calpains occurs in many neurodegenerative diseases and injuries to the CNS [4, 55, 126]. Increased calpain activity following TBI has been inferred by a variety of techniques [54, 81, 94, 97], including protection by calpain inhibitors [95, 109].

Pathological calpain activation is believed to occur when intracellular free calcium levels surpass a certain threshold. Importantly, increases in free calcium via voltage and receptor gated calcium channels have been reported in CNS trauma *in vivo* [19, 48, 56]. Calpains are located throughout the neuron, in somatodendritic regions and in axons [57]. Calpain may also be a constituent of myelin [116]. Therefore, pathological calpain activity and subsequent substrate proteolysis can have profound effects on neuronal structure and function.

Cytoskeletal alterations after experimental brain injury have pointed to the likelihood of calpain mediated proteolysis. Preferred substrates for calpains include the cytoskeletal protein spectrin [47, 66], microtubule associated protein-2 (MAP-2)[33], and neurofilament proteins [20, 21, 85, 105]. Increased degradation of MAP2 [32, 130], the neurofilament triplet proteins [53, 87] and spectrin [80] have been reported in cerebral ischemia. In addition, loss of MAP-2 [62], neurofilament 68 (NF 68) and neurofilament 200 (NF 200)[94-96] have been reported following traumatic brain injury (TBI) *in vivo*. Additional evidence that calpain is activated in neurons following experimental brain injury has been provided by the use of antibodies which bind specifically to calpain mediated BDP's of cytoskeletal proteins in models of TBI [81].

αII-Spectrin Degradation-A Prototype Biomarker

Our research program to develop biomarkers for TBI has focused on α-spectrin degradation as a prototypical biochemical marker [34, 103]. αII-spectrin is the major structural component of the cortical membrane cytoskeleton and is particularly abundant in axons and presynaptic terminals [36, 37]. Importantly, αII-spectrin is a major substrate for both calpain and caspase-3 cysteine proteases [124]. Our laboratory has provided considerable evidence that αII-spectrin is processed by calpains and/or caspase-3 to signature cleavage products *in vivo* after TBI [6, 11, 81, 92] and *in vitro* models of mechanical stretch injury [91]. Immunoblots of αII-

spectrin degradation thus provide concurrent information on the activation of calpain and caspase-3, potentially important regulators of cell death following TBI. The calcium sensitivity and low basal levels of calpain optimize its utility as a marker of cell injury. Although not found in erythrocytes and thus robust to confounding by blood contamination, αII-spectrin is not specific to the CNS [37]. Following injury, native αII-spectrin protein was decreased in brain tissue and increased in CSF from 24 hrs to 72 hrs after injury. Calpain-specific breakdown products increased in both brain and CSF after injury. Caspase-3-specific breakdown products increased in some animals, but to a lesser degree [90]. Considerable laboratory data exists on the potential utility of αII-spectrin degradation as a biomarker for TBI, including our ability to detect differences in severity of injury based on αII-spectrin detection in spinal fluid of rodents after TBI (Figure 1). Preliminary human data are also promising (Figure 2). However, what is not known is whether BDP's and other biomarkers that discriminate injury magnitudes at the biochemical level (i.e., magnitude differences in CSF/serum levels of a biomarker) will be useful as predictors of clinically relevant measures of outcome. That is, two different injury magnitudes may produce significantly different biochemical responses in the brain that are discernable in CSF or serum, but these same injury magnitudes may not result in functionally different behavioral, pathological, or other clinical outcome measures. Thus, biomarkers that do not correlate with clinically relevant outcome measures will not be useful for assessment of functional ability, functional recovery, or for gauging effects of therapy on outcome. In addition, preliminary results from our laboratories suggest that there is a spontaneous susceptibility of cytoskeletal protein degradation by calpain in aging rats [9]. These findings emphasize the importance of accounting for multiple clinical variables including but not limited to age when evaluating the clinical utility of biomarkers of brain injury.

Additional Cytoskeletal Proteins with Potential Utility as Biomarkers

Initial research focused on proteolytic processing of cytoskeletal proteins such as lower molecular weight neurofilament 68 protein (NF-68) highlights their potential to provide useful information on activity of specific proteases such as μ-calpain and m-calpain. Importantly, 2-D gel electrophoresis studies suggested dephosphorylation of NF-68 may be associated with NF protein loss following TBI, a post translational modification that could have significance for biomarker development [81, 97]. This important biomarker could provide important information on the pathophysiology of both dendritic and axonal damage after TBI [96]. Importantly, NF-68 has been used to quantify axonal injury in closed head injury models [102]. Since diffuse axonal injury (DAI) is presently considered one of the most common types of primary lesions in patients with severe closed head injury [88], a biomarker that provides information on axonal injury could potentially have clinical utility.

Future Directions

The pathology of TBI is extremely complex. As our understanding of the numerous biochemical cascades involved continues to evolve, sophisticated diagnostic tools such as biomarkers will be developed (Figure 3). Ideal biomarkers will provide information on the pathobiology of TBI and facilitate better stratification of patients by their severity of injury, better monitoring of the progression of secondary damage, response to treatment/intervention, and prediction of outcome. Although the initial characterization of biomarkers will be mainly based on spinal fluid analysis (Figure 3), methods for measurement of such biomarkers in blood (plasma or serum) will be developed. The development of accessible and reliable biomarkers is likely to change the way clinical studies of head injury are conducted, resulting in more mechanism driven, optimally timed therapies.

Acknowledgments

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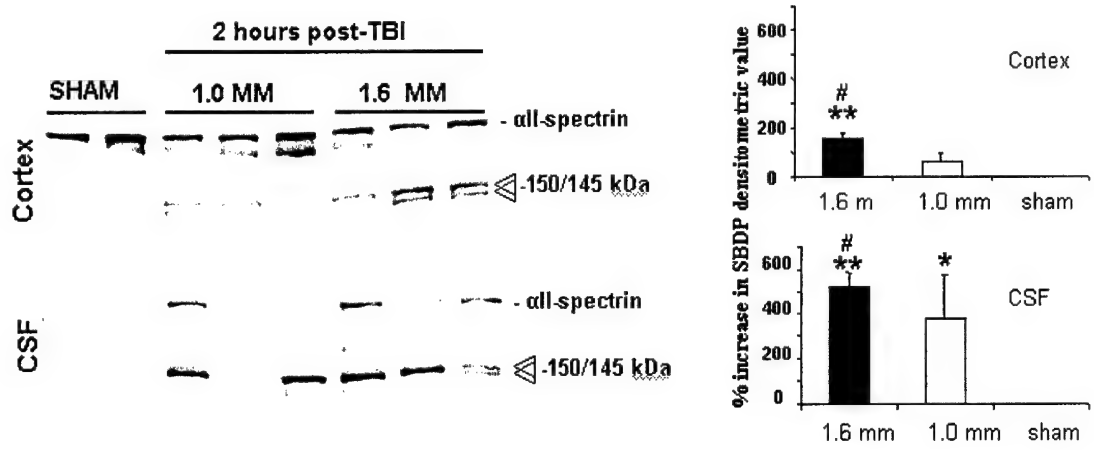

Figure 1. Accumulation of calpain-specific SBDPs is sensitive to injury magnitude after controlled cortical impact. Animals were injured using a model of controlled cortical impact at two magnitudes of injury. Accumulation of brain cortex and spinal fluid levels of αII-SBDP were measured by densitometric analysis at 2 hours post-injury. The 1.6 mm injury magnitude produced the highest levels of αII-SBDP accumulation in cortex and CSF compared to 1.0 mm injury and to sham-injured controls. However, statistical significance ($p<0.05$) between 1.6 mm and 1.0 mm injuries was only achieved at 2 hours but not at 6 or 24 hours post-injury (data not shown). This finding could indicate a critical window for discriminating injury magnitude using αII-SBDP as a biomarker. CSF levels of αII-SBDP were greater in CSF than in cortex, particularly for the 1.6 mm injury. This most likely reflects a greater ratio of SBDP to total protein in CSF relative to cortex and indicates that low abundance proteins may be more easily detected in CSF after brain injury. (n=6 per group).

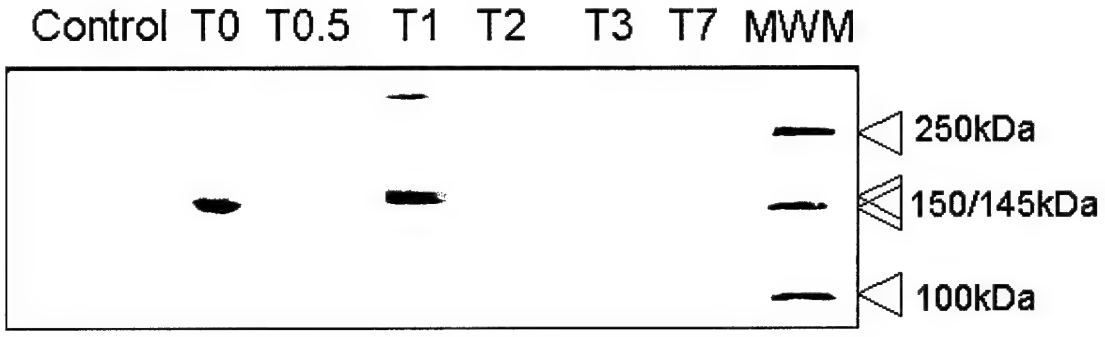

Figure 2. Western blot analysis of cerebrospinal fluid (CSF) samples from a 9 year old patient diagnosed with severe head injury. αII-spectrin breakdown products reflecting caspase-3 (150kDa and 120kDa bands) and calpain (150kDa and 145kDa bands) activity were identified as early as 12 hours after injury and persisted for at least 3 days after injury. Increased protein expression 1 day after injury may reflect clinical evidence of increased brain swelling and intracranial hypertension. CSF from a child with chronic hydrocephalus and no acute brain injury was used as control. T0= admission to the intensive care unit; T0.5= 12 hours after injury; T1= 24 hours after injury; T2: 2 days after injury; T3: 3 days after injury; T7= 7 days after injury; MWM= molecular weight marker.

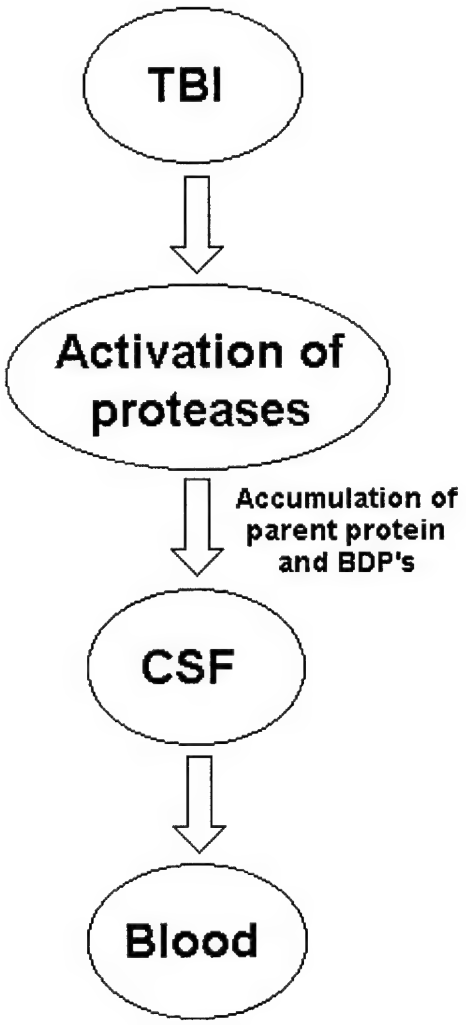

Figure 3. Development of biomarkers of protease activity. Brain injury leads to activation of proteases after traumatic brain injury. Proteolytic processing of cytoskeletal proteins (i.e. αII-spectrin) leads to accumulation of both the parent protein as well as signature break down products (BDP's). Initial characterization will most likely require analysis of cerebrospinal fluid (CSF). Antibodies will then be developed so that an easily accessible biomarker of protease activity is available for sampling from peripheral blood.

A NOVEL BIOMARKER FOR TRAUMATIC BRAIN INJURY:

CSF αII-SPECTRIN BREAKDOWN PRODUCT LEVELS ARE ASSOCIATED WITH INJURY MAGNITUDE AND PREDICT LESION SIZE

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Running title:
SBDP, a novel biomarker of Head Injury

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ABSTRACT

Currently there is no definitive diagnostic test for traumatic brain injury (TBI) to help physicians determine the seriousness of injury or the extent of cellular pathology. Calpain cleaves αII-spectrin into breakdown products (SBDP) after TBI and ischemia. Injury magnitude significantly elevated the mean levels of both ipsilateral cortex (IC) and cerebral spinal fluid (CSF) SBDP at 2, 6, and 24 hours after two levels of lateral controlled cortical impact (1.0 mm and 1.6 mm of cortical deformation) in rats. CSF SBDP levels were significantly higher after severe (1.6 mm) injury than mild (1.0 mm) injury. CSF SBDP levels were significantly correlated to IC levels in individual rats at 2, 6 and 24 hours after TBI. We also assessed the correlation between CSF SBDP levels and lesion size from T2-weighted magnetic resonance images (MRI) at 24 hours after TBI as well as correlation of two additional biomarkers, tau and S100β. Mean levels of CSF SBDP ($r = 0.833$) and tau ($r = .693$) significantly correlated with lesion size while levels of CSF S100β did not ($r = 0.188$). In a model to determine which marker or combination of markers (SBDP, tau, S100β) best predicted lesion size, CSF SBDP levels were the only significant predictor of lesion size. Furthermore, larger lesion sizes 24 hours after TBI were negatively correlated with decreased motor performance on days 1-5 after TBI ($r = -0.708$). Based on this data, we propose that CSF SBDP levels are a novel and promising biomarker of TBI and other acute CNS injuries.

Key Words: biomarker, CSF, injury magnitude, lesion size, spectrin, S100β, tau,

INTRODUCTION

The difficulty of diagnosis and prediction of outcome after acute traumatic brain injury (TBI) is associated with the limitations of clinical assessment and neuroimaging (Zink, 2001). Sedatives may be used to treat patients with TBI that exhibit confusion, agitation, or non-compliance with accompanying increased brain metabolism (Mirski *et al.*, 1995). Treatment with anti-convulsant or sedative drugs may confound information from a clinical neuropsychological examination (Mirski *et al.*, 1995). Many mild head trauma patients with a Glascow Coma Score (GCS) between 13-15 may have coincidental intoxication with drugs and alcohol which may also confound a clinical neuropsychological examination (Kelly, 1995). Head injuries may also be overlooked in multi-trauma patients (Buduhan and McRitchie, 2000). Clinical indicators may not predict significant intracranial trauma (Harad and Kerstein, 1992). Neurologic damage from traumatic brain injury (TBI), stroke or perinatal asphyxia may precede changes seen by modern neuroimagining techniques. Although mild traumatic injury may cause long term disabilities, mild trauma may not be seen acutely with radiologic or magnetic resonance imaging. Computed tomography (CT) scanning is the quickest and most available neuroimaging, yet has low sensitivity for diffuse brain damage . In a critical care patient, cost, availability, and time to acquire images limit the more sensitive measures of magnetic resonance imaging (MRI) and single photon emission CT scans. Single photon emission CT scans detect regional changes of blood flow but not necessarily structural damage. Furthermore, MRI and CT often do not predict outcome (Kido *et al.*, 1992; Kurth *et*

al., 1994; Wilson *et al.*, 1995; Hanlon *et al.*, 1999). There is thus a need for a biochemical marker of neuronal injury to improve diagnosis and prediction of outcome after TBI.

An ideal biomarker would incorporate several properties. A good biomarker would diagnose neurologic damage before neuro-radiographic signs are evident. A biomarker of acute neuronal injury should indicate injury magnitude and predict neuropsychological outcome. The biomarker should also reflect the pathogenesis of cell death including secondary cell death and indicate a target for treatment. With earlier recognition, the window for therapeutic intervention could be extended. Furthermore, a good biomarker would allow for longitudinal monitoring of the effectiveness of therapy. A biomarker with these characteristics could be used as a surrogate marker and lower the cost of clinical trials. An ideal biomarker should also be specific to the central nervous system and provide a sensitive and specific test of neuronal injury.

Earlier biomarkers such as neuron-specific enolase, lactate dehydrogenase, or creatine kinase have not been specific to the CNS, failed to reflect pathophysiology, or failed to predict lesion size and outcome further reinforcing the need for research into better CNS trauma indicators (Ingebrigtsen and Romner, 2002). S100β, a low molecular weight calcium-binding protein released from astrocytes, has been examined in numerous TBI studies. Serum levels of S100β correlated with contusion volume (Raabe *et al.*, 1998; Herrmann *et al.*, 2000); injury severity (Herrmann et al., 2000); neuropsychological dysfunction (Herrmann et al., 2001); GCS on admission (Elting *et al.*, 2000); and outcome measures such as the

Glascow Outcome Score (GOS) (McKeating *et al.*, 1998; Elting *et al.*, 2000; Jackson *et al.*, 2000; Raabe and Seifert, 2000; Rothoerl *et al.*, 2000). S100β appears to be a valuable indicator of brain lesion but it does not appear to reflect pathophysiology, nor is it specific to the central nervous system (CNS). Importantly in multitrauma patients without head injuries, S100β reached high serum levels after bone fractures and thoracic contusion (Anderson *et al.*, 2001). Another possible biomarker for brain injury is tau (Zemlan *et al.*, 1999), a microtubule associated protein expressed predominantly in axon of neurons and implicated in microtubule stability, axon elongation and axon transport (Garcia and Cleveland, 2001). In severe TBI patients, CSF levels of cleaved tau (c-tau) were significant predictors of intracranial pressure and GOS at discharge (Zemlan *et al.*, 2002), but in recent studies, CSF total tau levels did not correlate with GOS in patients with severe TBI (Franz *et al.*, 2003) nor did serum cleaved tau levels correlate with outcome measures (Chatfield *et al.*, 2002).

αII-spectin in the CNS is primarily localized to axons and to the presynaptic terminal of neurons (Riederer *et al.*, 1986). In acute neuronal injury, αII-spectin, a cytoskeletal protein, is a substrate for the calcium activated cysteine proteases, calpain (calpain-1 and –2) and caspase-3. After acute neuronal injury, calcium influx initiates a cytotoxic cascade of proteases, phospholipases, kinases and phosphotases including activation of calpain and caspases which results in necrotic and apoptotic cell death respectively. Calpain and caspase-3 both cleave the 280 kDa parent band of αII-spectrin into a 150 kDa breakdown product (SBDP 150). Calpain and caspase-3 cleave signature breakdown products of 145

(SBDP 145) and 120 kDa (SBDP 120), respectively, *in vivo* and *in vitro* (Nath *et al.*, 1996; Wang *et al.*, 1998; Wang, 2000). Both the calpain-mediated SBDP 145 and SBDP 150 increased acutely in the injured cortex whereas the caspase-3 mediated SBDP 120 was absent in an unilateral controlled cortical impact (CCI) model of TBI (Pike *et al.*, 1998). This may reflect a more prominent role of oncosis than apoptosis in the cortex in our CCI model.

αII-spectrin breakdown products (SBDP) have been used as an indicator of calpain activity in models of TBI (Newcomb *et al.*, 1997b) and ischemia (Bartus *et al.*, 1998). In our laboratory, levels of SBDP have recently been found to increase in rat CSF after experimental controlled cortical impact TBI (Pike *et al.*, 2001) and middle cerebral artery occlusion (Pike *et al* 2003). In this study we extend this work by systematically comparing CSF SBDP to their counterpart in injured cortex, to injury magnitude, to CSF tau and S100β and to lesion volume (accessed by MRI). This is the first study to subject a biomarker of CNS injury to rigorous preclinical validation of its utility as an indicator of acute neuronal injury. Based on the data we have obtained, we propose that CSF SBDP levels are a novel and promising biomarker of acute CNS injury.

METHODS

Animals

Three groups of adult male (280-300 g) Sprague-Dawley rats (Harlan; Indianapolis, IN) were used. For study 1, CSF was withdrawn from one group of 90 rats and they were sacrificed at 2, 6 and 24 hours after TBI. At each time point of 2, 6, and 24 hours, 9 rats received mild (1.0 mm of cortical deformation) injury, 9 rats received severe (1.6 mm of cortical deformation) injury, 8 rats received a craniotomy but no cortical deformation (sham injury) and 4 rats remained naïve (no craniotomy or cortical deformation). For study 2, a second group of rats were sequentially scanned by MRI, subjected to CSF withdrawal, and sacrificed at 24 hours. Of the second group, 9 rats each received severe (1.6 mm) injury, mild (1.0 mm) injury or sham surgery and 8 rats remained naïve. One CSF tap on a rat with severe injury appeared to be frank blood and was removed from the study because the amount of blood would dilute out the concentration of the marker in the CSF and might significantly introduce blood-born markers. For study 3, 35 rats were tested on the rotarod at days1- 5 after TBI and scanned by MRI at 24 hours and 28 days after TBI. Of the third group of rats, 10 rats each received severe (1.6 mm) injury, mild (1.0 mm) injury or sham surgery and 5 rats remained naïve.

Surgical Preparation and controlled cortical impact traumatic brain injury. As previously described (Dixon *et al.*, 1991; Pike *et al.*, 2001), a cortical impact injury device (CCI) was used to produce traumatic brain injury (TBI). Adult male rats were initially anesthetized with 4% isoflurane in a carrier gas of 1:1 O_2 / N_2 (4

min.) followed by maintenance anesthesia of 2.5% isoflurane in the same carrier gas. Core body temperature was maintained at 37±1°C by placing an adjustable temperature controlled heating pad beneath the rats. Animals were mounted in a stereotactic frame in a prone position and secured by ear and incisor bars. A midline cranial incision was made, the soft tissues were reflected and a unilateral (ipsilateral to site of impact) craniotomy (7 mm diameter) was performed adjacent to the central suture, midway between bregma and lambda. The dura mater was kept intact over the cortex. Brain trauma in rats was produced by impacting the right cortex (ipsilateral cortex) with a 5 mm diameter aluminum impactor tip (housed in a pneumatic cylinder) at a velocity of 3.5 m/s with a 150 ms dwell time (compression duration). Compression depth was set at 1.0 mm (mild), or 1.6 mm (severe). Velocity was controlled by adjusting the pressure (compressed N_2) supplied to the pneumatic cylinder. Velocity and dwell time were measured by a linear velocity displacement transducer (Lucas Shaevitz™ model 500 HR; Detroit, MI) that produces an analogue signal by a storage-trace oscilloscope (BK Precision, model 2522B; Placentia, CA). Sham-injured animals underwent identical surgical procedures for a craniotomy but did not receive cortical compression. Naïve rats did not undergo surgery or injury. Appropriate pre- and post-injury management was maintained to insure that all guidelines set forth by the University of Florida Institutional Animal Care and Use Committee and the National Institutes of Health guidelines detailed in the Guide for the Care and use of Laboratory Animals were complied with.

CSF Withdrawal

Under anesthesia, the rat was secured in the same stereotactic frame as used in surgery. The neck was flexed to optimize exposure of the atlanto-occipital space. A mid-line incision was made over the superficial cervical muscles. A 25 G needle attached to polyethylene tubing was inserted into the atlanto-occipital space and CSF was gently withdrawn. CSF was immediately spun at 9,000 g for 5 minutes at 4^0 C to remove any red blood cells from the cortical impact or from the tap. CSF was frozen at -80^0 until examined.

Tissue Lysis

Cortical tissues were collected from naïve animals or at 2, 6, and 24 hours after sham surgery or TBI. At the appropriate post-injury time-points, the animals were anesthetized with 4% isoflurane in a carrier gas of 1:1 O_2/N_2O (4 min.) and subsequently sacrificed by decapitation. Ipsilateral (to the impact site) cortex samples were rapidly dissected and snap-frozen in liquid nitrogen. Tissue samples were stored at -80°C until further processing. The samples were homogenized in a glass tube with a Teflon dounce pestle in 15 volumes of ice-cold detergent-free buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM EGTA, 0.33 M sucrose, 1 mM DTT) containing a broad-range protease inhibitor cocktail (Roche Molecular Biochemicals, #1-836-145) and sonicated. Samples were then centrifuged at 9000 g for 5 minutes at 4°C. The supernatant was stored at -80°C until immunoblot analysis.

Immunoblotting

Prior to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), protein content was assayed by the Micro BCA method (Pierce, Rockford, IL, USA) with albumin standards. From each sample, 40 μg of protein from cortical tissue or 40 μg of protein from CSF samples were added to 2X loading buffer containing 0.2 M Tris (pH 6.8), 400 mM 2-mercapto-ethanol, 8% SDS, 0.04% Bromophenol Blue, and 40% glycerol (1X loading buffer contains 125 mM Tris-HCl (pH 6.8), 100 mM 2-mercapto-ethanol, 4% SDS, 0.01% bromophenol blue, and 10% glycerol). The amount of protein loading for CSF samples was optimized to identify SBDP after both mild (1.0 mm) and severe (1.6 mm) injury. This would sometimes result in blurring of the 145-150 kDa spectrin breakdown products. The 145-150 kDa spectrin breakdown product represents primarily calpain initiated cleavage of spectrin in our model. Consistent with a previous report that CCI in our laboratory does not produce prominent caspase-3 levels (Pike et al., 1998), caspase-3 mediated SBDP 120 was inconsistent after severe (1.6 mm) injury and absent after mild (1.0 mm) injury and was not analyzed in this set of experiments. Semi-quantitation by densitometry evaluated both the 145-150 kDa band together thus the blurring was not a problem. Samples were heated at 96°C for 10 minutes and then centrifuged for one minute at 10,000g. Samples were resolved in a vertical electrophoresis chamber for 70 minutes at 150V. A 6.5% percent stacking acrylamide gel or a 4-20% Tris-Glycine gel (Invitrogen Life Technologies, Carlsbad, CA) were used. For the first part of the study, separated proteins were laterally transferred as a wet transfer to a nitrocellulose membrane

(0.45 μM) using a transfer buffer consisting of glycine (192 mM) and tris (25 mM), (pH 8.3) with 10% methanol at a constant voltage of 100V for 70 minutes at 4°C. For the second part of the study, separated proteins were horizontally transferred as a semi-dry transfer to an Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) using 39 mM glycine, 48 mM Tris, and 5% methanol at 20 V for 2 hours at room temperature. All gels were stained with coomassie blue to confirm equal loading of protein on the gel. For the first part of the study, membranes were also stained with Panceau red (Sigma, St. Louis, MO) to confirm transfer and equal amounts of protein were loaded in each lane. Blots were blocked for one hour in 5% non-fat milk in TBST (20 mM Tris, 0.15 M NaCl, and 0.005% Tween-20). Following overnight incubation with primary antibody, anti-α-spectrin monoclonal antibody (Affiniti Research Products, UK) (1:10,000 dilution for cortex and 1:5,000 dilution for CSF) and 1% non-fat milk/TBST at 4°C temperature, blots were incubated with goat anti-mouse antibody (Biorad) (1:1000 for cortex and 1:5000 for CSF) and 3% non-fat milk/TBST as secondary for 1 hour. Blots were then washed for one hour in TBST. Enhanced chemiluminescence reagents (ECL and ECL-Plus, Amersham) were used to visualize immunolabeling of cortical tissue and CSF respectively and developed on Kodak BioMax Light Film (Kodak). Semi-quantitative evaluation of protein levels detected by immunoblotting was performed via computer-assisted one-dimensional densitometric scanning (ImageJ, version 1.29, NIH, USA). Data was acquired as integrated densitometric values from similarly exposed films.

ELISA

CSF S100β levels were measured with a rat specific ELISA kit, Nexus D_x^{TM} Rat S100 Test Kit from SynX (Toronto, Ontario, Canada) and CSF tau was measured by a kit,Innotest^{TM} hTau Antigen from Innogenetics,Inc. (Alpharetta, GA, USA). Sensitivity of the S100β and tau ELISA kits was 0.02 ng/ml and 75 pg/ml respectively.

T2-weighted magnetic resonance imaging

Animals were scanned in the Advanced Magnetic Resonance Imaging and Spectroscopy (AMRIS) facility located in the McKnight Brain Institute of the University of Florida. Animals undergoing these imaging sessions were anesthetized with isoflurane (maintenance anesthesia of 1.5%-2.5% isoflurane in 1L/minute100% O_2 continuously delivered via a nose cone). Ophthalmic lubricant was placed in the eyes of all anesthetized animals. Anesthetized rats were placed on a custom Plexiglas cradle constructed to support the rat comfortably in the supine position. Oxygen saturation was monitored using a pulse oxymeter positioned on the left hind limb. Body temperature was monitored using a rectal flouroptic probe and maintained using warm air. A 4.7 Tesla magnet (Oxford Instruments) and Bruker Avance Console (Bruker, Germany) and a custom built 3.3 cm (inner diameter) quadrature birdcage coil were used for all image acquisition. T2-weighted images were acquired at 24 hours and 28 days after TBI. Twelve contiguous 1.25 mm coronal slices were acquired with the following parameters: a field of view = 3.6 x 3.6 cm^2, repetition time (TR) = 2.1 seconds,

echo time (TE) = 81 ms, matrix = 256x256 points per dimension (140 micron in plane). Areas of hypo-intensity on MRI were associated with hemorrhage or mechanical disruption and areas of hyper-intensity were associated with edema (Albensi et al., 2000). Lesion size was drawn using ParaVision Image Analysis tools (Bruker, Germany) similar to the methodology in (Neumann-Haefelin *et al.*, 2000). The area of each lesion in each coronal slice was multiplied by the slice thickness and then added to calculate the total lesion size.

Neurological functional evaluation

Rota-rod Test

Motor behavior was assessed in the sub-acute period after TBI by a blinded observer on a Rota-rod (Ugo Basile, Comerio, Italy)(Hamm *et al.*, 1994). Rats were placed on a Rota-rod, a rotating rod, which slowly accelerated from 4 to 40 rpm within 5 minutes. The Rota-rod requires the rat to walk as the revolving rod accelerates and maintain equilibrium. The trial lasted until the rat fell off and tripped a plate that records the time or until 300 seconds was reached. Rats underwent conditioning of two trials a day for three days prior to TBI. After TBI, the rats were tested for two trials a day on days 1-5. The average of the latency in seconds of the two trials was recorded.

Statistical analysis

Means and standard error of the means were calculated from individual rat densitometric values of the 145-150 kDa SBDP combined as one value. Two-way

ANOVA was used to examine main effects and interaction effects of time and injury magnitude. One-way ANOVA with contrast to do pair-wise comparisons was used to determine significance between levels of SBDP and between lesion sizes of the corresponding experimental groups. Regression analysis was performed with lesion size as the outcome variable and CSF markers (SBDP, tau, S100β) as the predictor variable. Pearson correlations were calculated and tested using the asymptotic Z-test. Repeated measures ANOVA *(4 groups X 5 time points)* were performed to determine individual group differences over the five time points on the Rota-rod test.

RESULTS

Injury magnitude is associated with increased levels of SBDP in the cortex and CSF after TBI.

SBDP were measured by western blot from the cerebral spinal fluid (CSF) and ipsilateral cortex (IC) at 2, 6, and 24 hours after two magnitudes of TBI. Naïve rats and sham rats that had undergone a craniotomy served as controls for this study. The two response variables, SBDP in the CSF and SBDP in the IC were analyzed via ANOVA with terms for injury magnitude, time, and the interaction of time and injury magnitude.

The results indicated there was no interaction effect ($p=0.88$) or time effect ($p = 0.12$) for on IC SBDP levels. The analysis also indicated that injury magnitude significantly increased the level of cortical SBDP ($p \leq 0.0001$). Mean levels of IC SBDP after severe (1.6 mm) injury were significantly higher than the mean levels of IC SBDP after mild (1.0 mm) injury ($p < 0.05$). Mean levels of IC SBDP after both severe (1.6 mm) and mild (1.0 mm) injury were significantly greater than mean levels of SBDP after sham-craniotomy or in naïve controls ($p < 0.0001$). Mean levels of CSF and IC SBDP did not differ between naïve and sham. Representative gels show that levels of SBDP increased with injury magnitude in the ipsilateral cortex and the CSF (**Fig. 1A**). Levels of SBDP (both 145 and 150 kDa are densitometrically quantified together) were highest after 1.6 mm injury in the IC and CSF at all time points (**Fig. 1B**).

After severe (1.6 mm) injury, the mean levels of IC SBDP were 116.4, 135.9, and 135.6 and after mild (1.0 mm) injury, the mean levels of IC SBDP reached 78.1, 110.1, and 102.8 at 2, 6, and 24 hours respectively. After sham-

craniotomy, the mean levels of IC SBDP reached 22.6, 40.9, and 11.8 at 2, 6, and 24 hours respectively. In naïve rats, the mean levels of IC SBDP were 4.4, 15.4, and 4.4 at 2, 6, and 24 hours respectively.

There was no interaction effect ($p = 0.39$) or time effect ($p = p = 0.13$) on CSF SBDP levels. The analysis also indicated that injury magnitude significantly increased the levels of CSF SBDP ($p \leq 0.0001$). Mean levels of CSF SBDP after severe (1.6 mm) injury were significantly higher than the mean levels of CSF SBDP after mild (1.0 mm) injury ($p = 0.0001$). Mean levels of CSF SBDP after both severe (1.6 mm) and mild (1.0 mm) injury were significantly greater than mean levels of CSF SBDP after sham-craniotomy or in naïve controls ($p < 0.0001$). Mean levels of CSF SBDP did not differ between naïve and sham.

After 1.6 mm injury, the mean levels of CSF SBDP were 153.4, 114.4, and 91.2 and after 1.0 mm injury, the mean CSF SBDP were 82.2, 71.4, and 64.3 at 2, 6, and 24 hours respectively. After sham-craniotomy, the mean levels of CSF SBDP reached 7.8, 19.1, and 10.3 at 2, 6, and 24 hours respectively. In naïve rats, the mean levels of CSF SBDP were 1.0, 7.7, and 4.18 at 2, 6, and 24 hours respectively.

Levels of SBDP in the CSF correlate with levels of SBDP in the ipsilateral cortex

CSF levels of SBDP were correlated to their counterpart levels in IC levels in individual rats at 2, 6 and 24 hours after TBI after sham-craniotomy, mild (1.0 mm) injury and severe (1.6 mm) injury and in naïve rats. CSF levels of SBDP

correlated with SBDP in the IC at 2 hours ($r = 0.728$), at 6 hours ($r = 0.690$), and 24 hours ($r = 0.574$) after TBI ($p < 0.01$) (**Fig. 2**).

CSF SBDP levels correlates with lesion size at 24 hours post injury

CSF extraction to measure SBDP and T2-weighted imaging to measure lesion size was performed in the same groups of rats at 24 hours after TBI. Representative T2-weighted images of a naïve rat and a rat 24 hours after sham-craniotomy, mild (1.0 mm) injury and severe (1.6 mm) injury are shown in **Fig. 3A**. Mean lesion size was significantly different between 1.6 mm and 1.0 mm injury groups and between both injury groups and the sham-injured group ($p \leq 0.001$). Severe (1.6 mm) injury resulted in disruption of normal architecture and swelling of the ipsilateral cortex (arrow in **Fig 3A**). Less disruption of normal architecture is noted after mild (1.0 mm) injury (arrowhead in **Fig 3A**). Sham-craniotomy resulted in varying amounts of hyper-intensity in the ipsilateral cortex (arrowhead in **Fig. 3A**). Average lesion size was 0.04447 $cm^3 \pm 0.00058$ after sham surgery, 0.10017 $cm^3 \pm 0.00996$ after 1.0 mm injury, and 0.16556 $cm^3 \pm 0.016095$ after 1.6 mm injury (**Fig. 3B**).

Mean levels of CSF SBDP significantly correlated with lesion size ($r = 0.833$, $p < 0.0001$) (**Fig. 4A**). To further explore the ability of SBDP to predict lesion size, a regression analysis was run with lesion size as the outcome variable and CSF SBDP as the predictor variable. The regression weight for CSF SBDP was estimated to be 1059.86, and the parameter estimate of the intercept was 10.707. The regression analysis revealed CSF SBDP contributed significantly to predicting lesion volume ($p < 0.0001$).

Levels of CSF tau significantly correlated with lesion size ($r = .693$, $p < 0.0001$) (**Fig 4B**) as levels of CSF S100β did not ($r = 0.188$) (**Fig 4C**). The regression weight for CSF tau was estimated to be 0.00001258, and the parameter estimate of the intercept was 0.03485. CSF levels of tau significantly contributed to the prediction of lesion volume ($p < 0.0001$).

A regression analysis was performed to determine which marker or combination of markers (SBDP, tau, S100β) best predicted lesion size. A full regression model indicated the only significant variable was SBDP ($p < 0.0001$). S100β was eliminated from the model and the regression re-run looking at SBDP and tau as predictors of lesion size. CSF SBDP was again the only significant predictor of lesion size ($p < 0.0001$). CSF SBDP and CSF tau are significantly correlated ($r = 0.750$, $p < 0.0001$) and CSF SBDP has a higher correlation with CSF tau than CSF tau's correlation with lesion size.

Injury magnitude is associated with decreased performance on the Rota-rod test and increased lesion size.

Because CSF SBDP correlated with lesion size at 24 hours, we looked at the relationship between lesion size and motor performance. Motor performance was assessed in the same (study 3) rats that lesion size was measured at 24 hours and 28 days (**Fig 5A**). Similar to 24 hours, at 28 days lesion size varied with injury magnitude. Lesion size at 24 hours in the individual animal was significantly correlated with lesion size at 28 days ($r = 0.881$, $p < .0001$). Assessment of Rota-rod performance prior to treatment revealed no significant differences between

groups. Injury magnitude had a significant effect on Rota-rod performance ($p < 0.0001$). Time did not significantly affect Rota-rod performance nor did time and injury magnitude interact to effect Rota-rod performance. Mean Rota-rod scores were significantly lower after 1.6 mm injury at all time points (1 – 5 days after TBI) compared to mild (1.0 mm) injury, sham-cranitomy or naïve rats ($p \leq 0.05$). After 1.0 mm injury, mean Rota-rod scores were significantly lower than in naïve rats ($p < 0.01$) and showed a trend toward being lower than after sham-craniotomy (**Fig.5A**). Naïve rats averaged close to a perfect score of 300 seconds at all time points. Naïve rats had significantly higher scores than rats after sham-craniotomy ($p < 0.05$). Furthermore, larger lesion sizes were associated with decreased performance on the Rota-rod (**Fig 5B**). In the individual rat, the average of the 5 days of Rota-rod scores correlated negatively with lesion size at 24 hours ($r = -0.708$; $p < 0.0001$).

DISCUSSION

This is the first paper to demonstrate CSF levels of SBDP have three properties of a good biomarker: 1) association with injury magnitude, 2) reflection of pathophysiology in the brain, 3) significant contribution to prediction of outcome as measured by lesion size. Not only do these studies strongly support the utility of CSF SBDP as a biomarker of acute neuronal injury, they provide further evidence of the relationship between injury magnitude and biochemical outcome measures. This study is also the first rigorous preclinical evaluation of a biomarker of acute neurological injury. The contribution of this work is a foundation for future studies assessing the utility of this marker in human brain injury.

Injury magnitude significantly increased CSF and cortical levels of SBDP over the two control groups, sham-craniotomy and naïve rats (**Fig. 1**). CSF levels of SBDP were significantly higher after severe (1.6 mm) injury than mild (1.0 mm) injury at 2, 6 and 24 hours after TBI reflecting injury magnitude (**Fig. 1B**).

Increased levels of calcium after TBI have been shown in several models (Fineman *et al.*, 1993; Nadler *et al.*, 1995; Verweij *et al.*, 1997; Xiong *et al.*, 1997). After TBI, calcium initiates a cytotoxic cascade of proteases including calpain which breaks down the cytoskeletal protein, spectrin. Higher levels of injury magnitude increased mRNA levels of calpain-1 and calpain-2 in the injured cortex and hippocampus (unpublished data). Similar to our study, varying injury magnitude by depth or by velocity of impact, significantly effected lesion size (Goodman *et al.*, 1994). Injury magnitude also significantly increased peak

intracranial pressure and hippocampal neuron loss in similar models of TBI (Cherian *et al.*, 1994; Goodman *et al.*, 1994). Temporal increases in intracellular calcium were correlated with injury magnitude after controlled cortical impact TBI in rats (Fineman *et al.*, 1993). The corresponding increase in calcium after more severe TBI may explain the association between injury magnitude and SBDP levels in the IC and CSF.

In the acute time period following TBI, CSF SBDP significantly correlated with cortical levels of SBDP and both increased with injury magnitude. Calpain-mediated SBDP have been extensively examined and shown to increase in *in vivo* and *in vitro* models of neuronal injury (Bartus *et al.*, 1995; Nath *et al.*, 1996; Saatman *et al.*, 1996a; Newcomb *et al.*, 1997a). Recently it has been shown that CSF SBDP increased in models of TBI (Pike *et al.*, 2001) and ischemia (Pike *et al.*, 2003). The increased levels of SBDP 150/145 are primarily associated with calpain activation in our CCI model. Although caspase-3 may also cleave spectrin to SBPD 150, similar to prior work in our laboratory (Pike et al., 1998), the caspase-3 signature SBDP 120 was not significant in our CCI model, suggesting a much less relevant role of caspase-3 in the production of SBDP in this model. Calpain inhibitors have been neuroprotective in models of TBI (Saatman *et al.*, 1996b; Buki *et al.*, 2003), ischemia (Bartus *et al.*, 1994; Hong *et al.*, 1994; Markgraf *et al.*, 1998), and spinal cord injury (Banik *et al.*, 1998). The ability of CSF levels of SBDP to reflect the pathophysiology of acute neuronal injury may provide a therapeutic target for treatment of TBI and an effective way to monitor treatment of TBI.

CSF levels of SBDP significantly contributed to prediction of lesion size after TBI. Other biomarkers have shown varying correlations with lesion size.

Serum levels of creatine kinase isoenzyme BB did not correlate with CT findings in patients with mild TBI (Levitt *et al.*, 1995). Two clinical studies of serum levels of S100β revealed a correlation with contusion volume (Raabe *et al.*, 1998; Herrmann *et al.*, 2000), while in a study of mild TBI, serum S100β levels did not correlate with MRI or CT scans (Herrmann *et al.*, 1999). S100β may be released from damaged glial cells, and this variable may not change consistently with the magnitude of injury.

Importantly in multi-trauma patients without head injuries, S100β reached high serum levels after bone fractures and thoracic contusion and also increased after burns and minor bruising (Anderson *et al* 2001). Numerous studies examined the use of S100β to mark cerebral damage after cardio-pulmonary bypass surgery (Ali *et al.*, 2000) but S100β was found to be released from the mediastinum of cardiopulmonary bypass patients (Anderson *et al* 2001). After stroke, higher serum S100β levels were associated with larger infarcts and more severe neuropsychological deficits (Aurell *et al.*, 1991; Abraha *et al.*, 1997; Buttner *et al.*, 1997). However Hill and colleagues (Hill *et al.*, 2000) found only 32% of stroke patients had elevated serum S100β on admission. Early identification of stroke is necessary for optimal treatment within three hours.

CSF c-tau levels were significant predictors of outcome measures (intracranial pressure and GOS at discharge) (Zemlan *et al.*, 2002) supporting the finding of a significant correlation between CSF tau and lesion size in our study. On the other hand, (Franz *et al.*, 2003) showed that CSF levels of total tau did not correlate with injury severity (initial GCS) nor with outcome (GOS). The wide

range of tau levels in that study was thought to be due to distance of the white matter lesion from the ventricles. Lesion variability is less in a model of CCI than in a clinical study of TBI. Initial examination of serum c-tau indicated the presence of serum c-tau increased the odds of an intracranial injury and a greater chance of a poor out-come (Shaw *et al.*, 2002), however, later work indicated serum cleaved tau levels did not correlate with outcome measures (Chatfield *et al.*, 2002). After acute stroke, tau increased in the CSF (Hesse et al., 2001) and serum (Bitsch *et al.*, 2002), and serum tau levels correlated to lesion size and severity. Similar to S100B, however, it increased in less than 50% of stroke patients (Bitsch *et al.*, 2002). Our study did not examine serum SBDP levels but further work will be important to establish if SBDP crosses the blood-brain barrier and reflects SBDP levels in the CSF and brain.

Changes in high resolution MRI have been shown to correlate well with histology in a lateral fluid percussion model (Albensi *et al.*, 2000) and a closed head injury model (Assaf *et al.*, 1997) of TBI. Areas of hypo-intensity on MRI were associated with hemorrhage or mechanical disruption and areas of hyper-intensity were associated with edema (Albensi *et al.*, 2000). Twenty-four hours after rats underwent sham-craniotomy, varying amounts of hyper-intensity were noted, most likely due to edema associated with the changes in cranial pressures. In the closed head injury model, areas of hyper-intensity decreased between 2 and 7 days after TBI likely representing resolution of edema (Assaf *et al.*, 1997). Similarly in our study, the overall size of the lesion decreased between 24 hours

and 28 days, although a significant correlation was maintained between lesion size in individual rats at the two time points.

This study examined *in vivo* lesion size and the correlation to neuromotor function (**Fig. 5B**). Higher levels of injury magnitude significantly increased lesion size and decreased motor performance. In a stroke model, lesion size from T2-weighted images at 2 and 7 days after ischemia was significantly correlated with an average of individual neurological score (Palmer *et al.*, 2001)). Similarly in our study, the larger the lesion size, the worse the performance on the motor function test. Because lesion size at 24 hours was highly correlated with lesion size at 28 days and significantly negatively correlated with motor performance, it is suggestive that acute levels of SBDP might correlate with both acute motor performance and chronic lesion size. Because withdrawal of CSF is a terminal procedure in our laboratory at this time, the correlation is only speculative.

In conclusion, the results of this study show that injury magnitude is associated with the levels of SBDP in the IC and CSF over acute time periods after TBI. We also showed the levels of CSF SBDP correlate with IC SBDP levels supporting the idea that CSF SBDP levels reflect the pathophysiology in the cortex at that time. We further showed that 24 hours after TBI, CSF SBDP significantly correlate with lesion size. In a model to determine which marker or combination of markers best predict lesion size, we found CSF SBDP levels to be the best predictor. αII-spectrin is not found in red blood cells (Pike *et al.*, 2001) although it is found in very low levels in other organs systems (Pike, Flint, Wang,

Hayes, unpublished data). Future work could confirm that CSF levels of SBDP have diagnostic utility for prediction of outcome after TBI in humans.

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Figure 1. Injury magnitude increases levels of SBDP in the ipsilateral cortex (IC) and CSF. A) A representative western blot of αII-spectrin and SBDP in the IC (left) and CSF (right) at 24 and 2 hours respectively after TBI. Samples were collected after severe (1.6 mm) injury, mild (1.0 mm) injury, sham-craniotomy or from naïve rats. Higher levels of SBDP are seen after severe (1.6 mm) injury than after mild (1.0) injury. Minimal SBDP is seen in the IC or CSF of naïve rats or after sham-craniotomy in rats. **B)** SBDP levels (145-150 kDa fragments) in the IC (left panel) and CSF (right panel) after sham-craniotomy, mild (1.0 mm) injury and severe (1.6 mm) injury at 2, 6, and 24 hours were quantified using computer-assisted densitometric analysis (ImageJ, version 1.29x, NIH, USA). Values from naïve animals were averaged as a separate time point. At each time point of 2, 6, and 24 hours, 9 rats received severe (1.6 mm) injury, 9 rats received mild (1.0 mm) injury, 8 rats received a sham-craniotomy and 4 rats remained naïve. An ANOVA was performed followed by contrast with pair-wise comparisons. Data is presented as the mean plus standard error. Standard error bars on the shams are present but not easily visable. Injury magnitude significantly increased mean levels of IC and CSF SBDP over time ($p < 0.0001$). Mean levels of SBDP after severe (1.6 mm) injury were significantly higher from the mean levels of SBDP after mild (1.0 mm) injury (tt $p = 0.0001$ and t $p < 0.05$ respectively for CSF and IC levels of SBDP). Mean levels of IC and CSF SBDP after both severe (1.6 mm) and mild (1.0 mm) injury were significantly greater than mean levels of SBDP after sham-craniotomy or in naïve controls (**$p < 0.0001$). Mean levels of CSF and IC SBDP did not differ between naïve and sham.

Figure 2. CSF levels of SBDP (145-150 kDa fragments) correlate with IC SBDP at 2, 6 and 24 hours after TBI. After SBDP levels were semi-quantified using computer-assisted one-dimensional densitometric scanning (ImageJ, version 1.29x, NIH, USA), CSF levels of SBDP were correlated to IC levels in individual rats at 2, 6 and 24 hours after sham-craniotomy, mild (1.0 mm) injury and severe (1.6 mm) injury and in naïve rats. A) At 2 hours after TBI, correlation between levels of SBDP in the CSF and IC = 0.728, $p \leq .01$. B) At 6 hours after TBI, correlation between levels of SBDP in the CSF and IC = 0.690, $p \leq 0.01$. C) At 24 hours after TBI, correlation between levels of SBDP in the CSF and IC = 0.574, p = 0.01). ■ = rats after 1.6 mm injury; ♦= rats after 1.0 mm injury, H rats after sham-craniotomy; Δ = naïve rats.

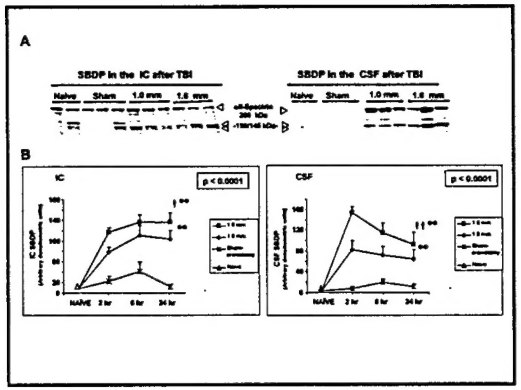
Figure 3. Lesion size on T2 weighted images increases with injury magnitude 24 hours after TBI. A) Representative serial T2-weighted magnetic resonance images of a naïve rat and a rat 24 hours after sham-craniotomy, mild (1.0 mm) and severe (1.6 mm) injury are shown. Twelve contiguous coronal 1.25 mm slices were acquired with the following parameters: a field of view = 3.6 x 3.6 cm^2, repetition time (TR) = 2100 seconds, echo time (TE) = 81 ms, matrix = 256x256 points per dimension (140 micron in plane). Four of the 12 coronal slices for each rat are shown. Severe (1.6 mm) injury resulted in disruption of normal architecture and swelling of the ipsilateral cortex (**arrow**). Less disruption of normal architecture is noted after mild (1.0 mm) injury (**arrowhead**). Sham-craniotomy resulted in varying amounts of hyper-intensity in the ipsilateral cortex

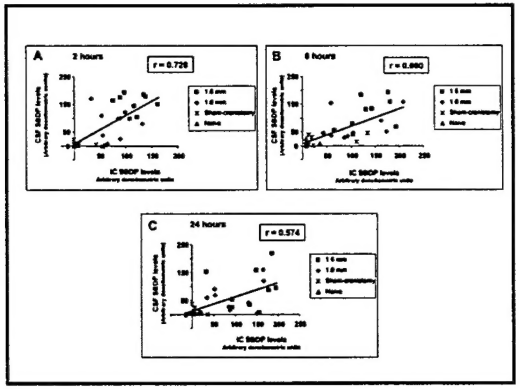
(**arrowhead**). **B)** Lesion size was drawn using ParaVision Image Analysis tools (Bruker, Germany) similar to the methodology in (Neumann-Haefelin *et al.*, 2000). The area of each lesion in each coronal slice was multiplied by the slice thickness and then added to calculate the total lesion size. One-way ANOVA with contrast to do pair-wise comparisons was used to determine difference between lesion sizes of the treatment groups. Nine rats each received severe (1.6 mm) injury, mild (1.0 mm) injury or sham surgery and 8 rats remained naïve. The lesion after severe (1.6 mm) injury is significantly greater than the lesion size after mild (1.0 mm) injury (# # = $p \leq 0.001$) and both are significantly greater than after sham-craniotomy (†† = $p \leq 0.001$).

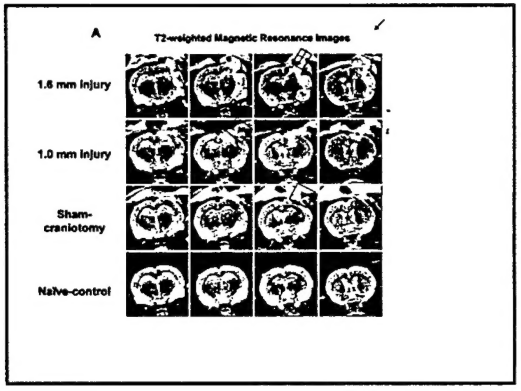
Figure 4. Levels of CSF SBDP and tau correlate with lesion size 24 hours after TBI, but CSF S100β does not. Regression analysis was performed with lesion size as the out-come variable and levels of CSF markers (SBDP, tau, S100β) 24 hours after TBI as the predictor variable. **A)** Levels of CSF SBDP correlate with lesion size after TBI ($r = 0.83$, $p \leq 0.0001$). A linear regression equation showed that CSF SBDP significantly contributed to prediction of lesion size ($p \leq 0.0001$). **B)** Levels of CSF tau correlate with lesion size after TBI ($r = 0.648$, $p < 0.001$). A linear regression equation showed that CSF tau significantly contributed to prediction of lesion size ($p \leq 0.0001$). **C)** Levels of CSF S100β did not correlate with lesion size ($r = 0.188$). ■ = rats after 1.6 mm injury; ♦= rats after 1.0 mm injury, H rats after sham-craniotomy; Δ = naïve rats.

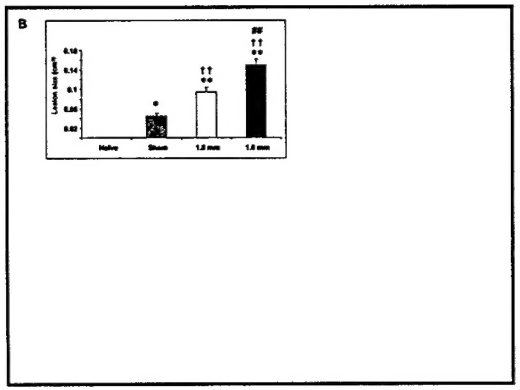
Figure 5. Performance on the Rota-rod test decreases with increased injury magnitude and lesion size.

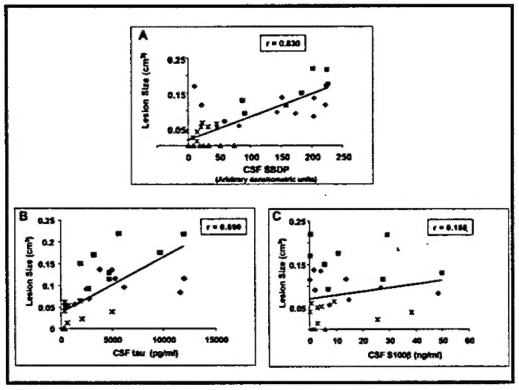
A) Rats were placed on a rotating rod, which slowly accelerated from 4 to 40 rpm with in 5 minutes on days 1-5 after TBI. The rats were tested for two trials a day and the average of the latency in seconds of the two trials was recorded. Repeated measures ANOVA (4 groups X 5 time points) were performed to determine individual group differences over the five time points on the Rota-rod test. Of the third group of rats, 10 rats each received severe (1.6 mm) injury, mild (1.0 mm) injury or sham surgery and 5 rats remained naïve. Data is presented as the mean ± the standard error. Injury magnitude significantly effected rotarod performance ($p < 0.0001$). Severely (1.6 mm) injured rats performed significantly worse on days 1-5 after TBI on the Rota-rod test than mildly (1.0 mm) injured rats or the sham-craniotomy group ($p < 0.05$ and $p < 0.01$ respectively). Both severe and mild injured groups performed significantly worse than the naïve rats ($p < 0.01$). **B)** Rotarod scores were averaged for the 5 days of testing for each individual rat. The average performance on the Rota-rod test was negatively correlated with lesion size at 24 hours after TBI in the individual rat ($r = - 0.708$; $p \leq 0.0001$). ■ = rats after 1.6 mm injury; ♦= rats after 1.0 mm injury, H = rats after sham-craniotomy; Δ = naïve rats.

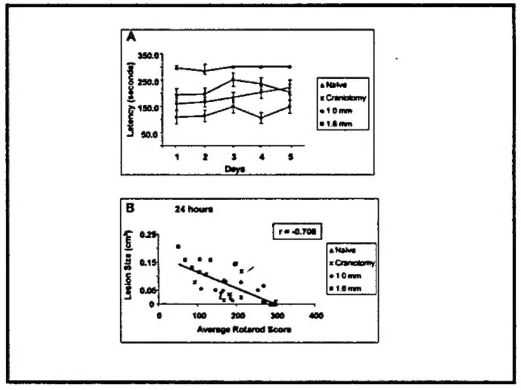

A
SBDP in the IC after TBI
SBDP in the CSF after TBI
Naive
Sham
1.0 mm
1.6 mm
B
IC
CSF
p < 0.0001
p < 0.0001

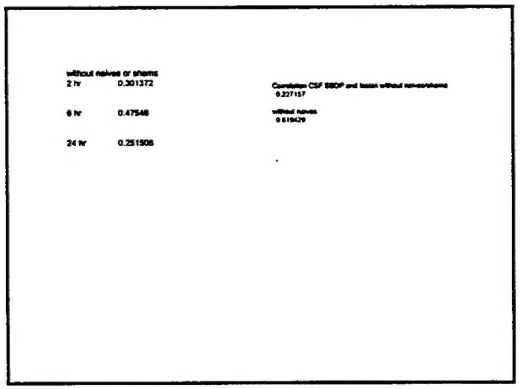

A
2 hours
r = 0.726
B
6 hours
C
24 hours
r = 0.574


A
T2-weighted Magnetic Resonance Images
1.6 mm injury
1.0 mm injury
Sham-craniotomy
Naïve-control


B
Naive
Sham
1.0 mm
1.6 mm


A
r = 0.630
CSF SBDP
B
r = 0.598
CSF tau (pg/ml)
C
r = 0.186
CSF S100β (ng/ml)


A
Latency (seconds)
Days
Naive
Craniotomy
1.0 mm
1.6 mm
B
24 hours
r = -0.708
Average Rotarod Score


without naives or sherms
2 hr 0.301372
6 hr 0.47548
24 hr 0.251906
0.227157
0.619429